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UNIVERSITY OF ALBERTA

TYROSINE TRANSPORT IN A HUMAN MELANOMA CELL LINE

BY

JAMES MILAN PANKOVICH

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

IN

Experimental Medicine

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Edmonton, Alberta

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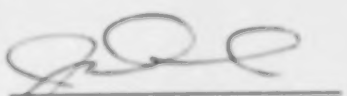

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Tyrosine Transport in a Human Melanoma Cell Line submitted by James Milan Pankovich in partial fulfillment of the requirements for the degree of Master of Science in Experimental Medicine.

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ABSTRACT

Tyrosine is an amino acid essential for the initial step of melanin synthesis, yet little is known concerning its transport in melanocytes. As an important first step in the development of new anti-melanoma agents based upon exploitation of the melanin synthetic pathway, this study characterized the transport mechanism of tyrosine *in vitro* using the human melanoma cell line, SK-MEL 23. Several tyrosine transport systems may be involved in melanocytes, systems L, T, $B^{o,+}$, and $b^{o,+}$ which transport neutral amino acids with branched or aromatic side chains, and systems A, ASC, and asc which transport neutral amino acids with smaller side chains. In order to determine which system or combination of these is involved in tyrosine transport by melanoma cells, kinetics, Na^+ dependence, and competitive inhibition studies were undertaken. The K_m and V_{max} for the Na^+-independent transport system were found to be 0.164 ± 0.016 mM and 21.6 ± 1.1 nmol/min/mg protein (mean ± SE) respectively. This transport was preferentially inhibited by the system L specific analog, 2-aminobicyclo-(2.2.1)-heptane-2-carboxylic acid (BCH), the system T substrate tryptophan, and the sulfur homolog of tyrosine, 4-S-cysteinylphenol (4-S-CP). Sequential addition of these inhibitors at increasing concentrations indicated that they may inhibit the same transporter.

Our results suggest that tyrosine transport in the SK-MEL 23 melanoma cells is similar to system L transport previously characterized in other cell types. This transport system appears to supply all the tyrosine required for both cell growth and melanin synthesis. The transport system may be subject to manipulation by melanogenic stimulating factors making the transport of cytotoxic tyrosine analogs an important area for further study.

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List of Abbreviations

UV	- ultraviolet light
5,6-DHI	- 5,6-dihydroxyindole
5,6-DHICA	- 5,6-dihydroxyindole-2-carboxylic acid
5-S-CD	- 5-S-cysteinyldopa
dopa	- 3,4-dihydroxyphenylalanine
MSH	- melanocyte stimulating hormone
cAMP	- cyclic adenosine monophorphate
4-S-CP	- 4-S-cysteinylphenol
4-S-CAP	- 4-S-cysteaminylphenol
PTU	- phenylthiourea
BCH	- 2-aminobicyclo-(2.2.1)-heptane-2-carboxylic acid
AIB	- 2-aminoisobutyric acid
MeAIB	- 2-methylaminoisobutyric acid
MEM	- minimal essential medium
FBS	- fetal bovine serum
EDTA	- ethylenediaminetetraacetic acid
dpm	- disintegrations per minute
BSA	- bovine serum albumin
HEPES	- N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
TPA	- 12-O-tetradecanoylphorbol 13-acetate

CHAPTER 1
INTRODUCTION

1.1 MELANOCYTES AND PIGMENTATION

Melanocytes are cells capable of synthesizing the enzyme tyrosinase which, when incorporated within specialized intracellular organelles, the melanosomes, initiates events leading to the synthesis and deposition of melanin (1). This definition of the metabolic machinery leading to melanin synthesis highlights the various unique features of the melanocyte. Those unique features may provide the basis for biological and physiological studies of properties related to the transport and regulation of substrates necessary for the synthesis of the pigments. The same features may provide the basis for establishment of specific chemotherapy of the neoplastic form of melanocytes, melanoma cells, in as much as this enzyme-substrate interaction could generate cytotoxic intermediates.

Embryologically melanocytes arise from the neural crest (Figure 1.1). In humans, melanocytes are present in the skin at the dermo-epidermal junction, in hair follicles and to a lesser extent in the lower layer of the skin, the dermis (2). In addition, melanocytes are

also found in the nervous system, eye, and inner ear where they are located in the dermis or among the connective tissues.

Melanocytes of the dermo-epidermal junction and hair follicles are responsible for the production of melanin pigments that will be a major factor in determining the color of skin and hair. Melanocytes discharge their melanin pigment granules into the surrounding keratinocytes to give the skin or hair visible color. Most of the extracutaneous melanocytes are comparable to dermal melanocytes in that they do not discharge melanin pigments into surrounding cells, but rather retain the pigments.

Normal human skin color - red, yellow, brown and blue - is produced by four skin pigments: by exogenously produced carotenoids (yellow) and endogenously produced melanin (brown) in the epidermis, by oxygenated hemoglobin (red) in the capillaries, and by reduced hemoglobin (blue) in the venules of the dermis (3). Of these, melanin is the major determinant of differences in skin color between individuals (3). It is the amount of melanin that has been transferred to the keratinocytes from the melanocytes that significantly determines the pigmentation of the skin and hair.

Melanin pigmentation of human skin can be divided into two components (2). Constitutive skin color is the

amount of cutaneous melanin pigmentation generated according to cellular genetic programs without any direct influence of external factors such as solar radiation. Facultative skin color or "tan" is elicited by direct exposure of the skin to ultraviolet (UV) light, or other regulators of melanin synthesis. Facultative skin color is reversible in that the skin responding to the stimulus will return to the original color upon removal of the stimulus. These color changes characterize the immediate and delayed tanning reactions induced by exposure to UV light.

1.2 MELANINS

There are two major classes of integumentary melanin formed by the action of tyrosinase: the black-brown nitrogenous eumelanin and the yellow to red, sulfur containing pheomelanin (4,5). Despite the obvious color differences these pigments are biologically related, and both arise from a common metabolic pathway in which tyrosine is the initial substrate and dopaquinone is the key intermediate (Figure 1.2).

Eumelanin is best characterized as a highly irregular, three-dimensional polymer of several types of monomers joined covalently into an integrated structure (1,5). The most common monomers are 5,6-dihydroxyindole (5,6-DHI) and 5,6-dihydroxyindole-2-carboxylic acid (5,6-

DHICA). Eumelanin has the characteristic of being insoluble in almost all solvents.

Pheomelanin appears in nature by a modification of the eumelanin pathway which involves the interaction of the amino acid, cysteine, or the tripeptide, glutathione, with dopaquinone, an intermediate produced by enzymatic oxidation of tyrosine. Chemically, pheomelanin is distinguishable by its relatively high content of sulfur which results from the addition of cysteine to dopaquinone to yield a 5-S-cysteinyldopa (5-S-CD) compound (1,4,5). This 5-S-CD monomer can then polymerize to form the yellow-red pigment.

Pure pheomelanin is formed only by cysteinyldopa oxidation products and pure eumelanin is formed only by indoles. In nature, these pure melanin products may be quite rare. Instead, most pigments seem to be mixed-type melanins which are formed by copolymerization of oxidation products of 3,4-dihydroxyphenylalanine (dopa) alone and dopa plus cysteine (1). The quantities of the intermediates available at the site of polymerization will determine the type of melanin formed. As well, melanin is not simply a polymer of oxidized tyrosine, but may contain proteins of undefined structure and function. These include structural proteins of the pigment granules and other proteins that are present in the melanosomes (5).

There is a wide range of physiological roles suggested for the melanins including protection of underlying tissues from UV radiation, heat control, and adaptive coloration (4,6). These may be evolutionarily conserved functions of a process that originally might have had a more fundamental biological significance than the provision of a visible pigment.

As early as 1820, Sir Everard Home first proposed a concept that melanin acts as a sunscreen (6). Today most pigment cell biologists believe that the primary function of melanin pigments in humans is as a sunscreen to protect the skin from the UV radiation in sunlight even though melanin may not fulfill all the properties of an ideal sunscreen.

1.3 MELANOSOMES

Within melanocytes, melanin is formed by the tyrosine-tyrosinase reactions and subsequent cyclizations of the products within intracellular organelles, the melanosomes. The formation of melanosomes involves the assembly and organization of four basic components that include membranous phospholipids, structural proteins, the essential enzyme tyrosinase, and possibly auxiliary enzymes (Figure 1.1).

Four stages of melanosome development are recognized in both eumelanogenesis and pheomelanogenesis (2). Stage

I melanosomes in eumelanogenesis are spherical vacuoles containing amorphous, proteinaceous materials and a few microvesicles also termed vesiculoglobular bodies. Stage II eumelanosomes take on a characteristic ellipsoidal shape and are assembled with organized lamellae of filaments and vesiculoglobular bodies. Tyrosinase activity is not detectable with the granules of stage I and II melanosomes by conventional techniques. In stage III eumelanosomes, tyrosinase activity becomes detectable and melanization begins on the lamellae of the inner matrix. Stage IV eumelanosomes are highly melanized and electron dense under electron microscopic examination.

Pheomelanosomes are always spherical and do not form a lamellar or fibrillar matrix such as seen in eumelanosomes. Instead, they conjugate together with numerous vesiculoglobular bodies or microvesicles in all stages of melanosomal development. The difference in morphology between pheomelanosomes and eumelanosomes may simply be based on a different organization of the same ingredients under the influence of sulfhydryl compounds (2).

1.4 TYROSINASE

Tyrosinase is a copper containing enzyme responsible for the production of melanin pigments from the initial substrate tyrosine. There has been a continuing

controversy whether there exists one enzyme with two functions or a two enzyme system, ie., tyrosinase plus another oxidation enzyme, for melanin synthesis. Evidence now seems to indicate that only one enzyme with two functions needs to be present to produce melanin (3,7). This single enzyme catalyzes the hydroxylation of tyrosine to dopa and subsequently the oxidation of dopa to dopaquinone. Melanin can be formed from dopaquinone without further enzyme participation. More recently, an enzyme, dopachrome conversion factor, has been identified that may act downstream from tyrosinase to increase the efficiency of the polymerization process (8).

Tyrosinase is initially transcribed on membrane bound ribosomes, transported through the endoplasmic reticulum to the Golgi region, where it is modified, packaged into vesicles, and then transported to melanosomes (Figure 1.1) (1). These tyrosinase containing vesicles then fuse with the melanosomal membrane to produce a stage III melanosome. The tyrosinase becomes activated in an as yet unknown manner, and melanin formation and deposition can proceed.

Tyrosinase can be present in multiple forms, T_1, T_2, T_3, all possessing tyrosine hydroxylase and dopa oxidase activity (4,9,10). These different forms probably represent the same core molecule at different stages of post-translational glycosylation (1,9). The core protein

has an apparent molecular weight of 55 kDa, and remains kinetically unchanged during its subsequent processing. Post-translational modifications may be necessary to assure the delivery and orientation of tyrosinase in the melanosome (9).

1.5 MELANOGENESIS

Having introduced the major components involved in melanin synthesis, it is important to understand how they all are integrated in a biological system to produce melanin pigments.

Even though many of the details of melanin synthesis are still to be worked out, it seems that the rate-limiting step in melanogenesis occur at the level of the oxidation of tyrosine to dopa. This conversion of tyrosine to dopa occurs inside the melanosomes and is mediated by the enzyme tyrosinase. Both types of melanin, eumelanin and pheomelanin, share this common step in the metabolic pathway (Figure 1.2). Eumelanin will be produced if dopaquinone proceeds to cyclize and oxidize. If, during the conversion of tyrosine to melanin, dopaquinone encounters cysteine or other sources of sulfhydryl groups, pheomelanin will be the major product.

The process of melanin synthesis is under the control of multiple genes, with more than 150 loci having

been implicated in mice. Thus, there are potentially many factors that can affect the rate and type of melanin synthesis.

Traditionally, melanocyte stimulating hormone (MSH) has been used to increase melanization of pigment producing cells in vitro (11,12). The increased melanization in response to MSH is mediated through the intracellular second messenger cyclic adenosine monophosphate (cAMP) (7,11). MSH is able to bind to receptors present on the cell surface and the formation of this receptor complex stimulates the activity of adenylate cyclase, which in turn leads to increases in the intracellular levels of cAMP.

MSH receptors are the best characterized regulatory elements governing pigmentation, morphology, and proliferation of pigment cells in vertebrates (13). The cells regulate their response to MSH by the transient appearance of cell membrane receptors for this hormone signal (7). An increase in tyrosinase activity can be detected 8 hours after MSH stimulation, and melanin formation takes place about 16 hours after the initial MSH challenge (12). MSH can also modify cellular proliferation and alter the morphology of melanoma cells in culture.

Exposure of melanocytes to an external regulator, UV radiation, leads to changes in melanin synthesis, the so

called tanning reactions (14-16). UV radiation results in an increase in melanocyte number by an undefined molecular mechanism and, subsequently the overall rate of synthesis of the enzyme (the long term effect). It also has a short term effect, ie., the rapid polymerization of pre-existing melanin precursors within the melanosome which occurs within a matter of minutes. Both of these effects result in overall increased pigment production. The UV radiation-mediated activation of melanogenesis has been found to be due to the activation of cAMP-independent mechanisms, perhaps involving the inositol triphosphate or diacylglycerol second messenger pathways (14,15,17).

There is recent evidence that, at least in vertebrates, tyrosine and dopa themselves can act as inducers and regulators of the melanogenic apparatus, the expression and activity of MSH receptors, and melanocyte proliferation (13,18-20). Supplementation of the growth medium with tyrosine or dopa during the culturing of amelanotic melanoma cells results in a rapid increase in melanin formation, which is not simply due to greater availability of substrate (18,19). Positive regulation of melanogenesis-related proteins following addition of tyrosine has been shown (20). Upon treatment with tyrosine in the culture medium there is a rapid increase in tyrosinase activity and large scale synthesis of

melanosomes. These two events would combine to increase the synthesis of melanin.

1.6 RATIONAL CHEMOTHERAPY

Malignant melanoma is a cancer with increasing worldwide incidence (21). At the present time, the primary treatment for localized melanoma is surgical excision. Prognosis worsens as the disease spreads with the median survival after diagnosis of metastasis being less than 5 months (22). Radiation and existing methods of chemotherapy are limited in their efficacy. Consequently there is an urgent need for new and improved treatments for malignant melanoma.

Since 1963 when Hochstein and Cohen (23) proposed that polyphenolic intermediates in the formation of melanin from tyrosine are potentially cytotoxic agents, many attempts have been made to try and exploit this biochemical pathway for the treatment of melanoma. Most attempts to develop chemotherapeutic agents specific to malignant melanoma have involved the exploitation of intermediates of melanin synthesis, agents which are assumed to enhance melanin pigmentation, or a combination of these two.

Ideally a chemotherapeutic agent should selectively discriminate between the normal and target neoplastic cell type to minimize the destruction of normal cells

essential for the survival of the host. This principle may not necessarily apply to melanoma chemotherapy. Melanocytes serve an apparently non-essential role in the host, therefore the destruction of normal melanocytes along with melanoma cells would be insignificant when compared to the manifestations of the disease itself. The specificity is important is being able to distinguish between melanin and non-melanin producing cells.

Melanocytes and melanoma cells both contain the unique enzyme tyrosinase. This enzyme is capable of catalyzing the hydroxylation of tyrosine to dopa and the oxidation of dopa to dopaquinone. These are the first two steps in the melanin synthetic pathway. The enzyme tyrosinase provides these melanin producing cells with a specific biochemical target to exploit in the development of chemotherapeutic agents. Since this enzymatic activity is restricted to melanocytes and generally present in larger amounts in melanoma cells (24), a defined biochemical target is present. The rationale of this approach is not necessarily to interfere with melanin biosynthesis since melanin production is not essential to the general metabolic economy of the melanoma cell, but rather to design compounds which are converted to cytotoxic derivatives by the action of a unique enzymatic apparatus within the melanoma cell. It is known that even some of the natural melanin precursors

and intermediates are cytotoxic and that cells actively producing melanin run the risk of self-destruction through a build up of these precursors (25).

Numerous melanin precursors have been explored as possible chemotherapy agents. These may be grouped into one of three categories and considered to be related to either tyrosine (phenolic), 3,4-dihydroxyphenylalanine (catecholic), or 5,6-dihydroxyindole (intermediates).

Tyrosine serves as the starting material for the biosynthesis of melanin and is also an important substrate for melanoma cells in vitro. Therefore, in vitro the degree of pigmentation can be influenced by changing the concentration of tyrosine in the growth medium. Analogs of the amino acid tyrosine have been developed as potential chemotherapeutic agents. This group of phenolic melanin precursors include the sulfur homolog of tyrosine, 4-S-cysteinylphenol (4-S-CP), its amine derivative, 4-S-cysteaminylphenol (4-S-CAP) (26-28), and 4-S-CAP derivatives (28,29). Both 4-S-CP and 4-S-CAP have been tested and shown to increase the life span of melanoma bearing mice and to cause the in vitro growth inhibition of melanoma tissue (27-30). Subcutaneous injections of 4-S-CP and 4-S-CAP into C57BL/6J black mice caused the depigmentation of the black hair (31), and topical application of these compounds to black guinea pigs resulted in depigmentation

of the skin (32). In both cases, the depigmentation was manifested by the loss of functioning melanocytes.

3,4-Dihydroxyphenylalanine (dopa) and its structural analogs are also known to be cytotoxic to melanoma cells. Dopa, dopamine, dopa methyl ester, norepinephrine, 6-hydroxydopamine, and 3,4-dihydroxybenzylamine were studied for their effects on human and murine melanoma cells *in vitro* (33,34). Both dopa and dopamine analogs are capable of inhibiting the growth of a variety of cell lines.

Two mechanisms have been proposed concerning the cytotoxicity of catechols. The first is an intracellular mechanism in which catechols are converted to the corresponding benzoquinones by tyrosinase, and these quinones are then believed to cause damage to cells through inactivation of sulfhydryl enzymes essential for cellular function (34,35). An alternative mechanism involving catechols has them being auto-oxidized extracellularly by the interaction with amine oxidases to produce cytotoxic hydrogen peroxide, superoxide, and hydroxyl radicals (33,34,36-38). This second mechanism can result in systemic rather than specific toxicity, decreasing the efficacy of the compounds as chemotherapeutic agents. The major limitations of dopa derivatives appear to be: 1) instability, 2) insufficient lethality of dopa toward neoplastic cells *in*

vivo, and 3) low physiologically tolerable dosages of 6-hydroxydopa and other catechols due to general cytotoxicity (26).

Intermediates in the melanin synthetic pathway are also potential chemotherapeutic agents. 5,6-Dihydroxyindole, an intermediate in the eumelanin pathway, has been shown to be toxic to melanoma cells in vitro (39). The catecholic amino acid, 5-S-CD, a major intermediate of pheomelanin, is also toxic to a variety of human tumor cell lines including murine L1210 leukemia and B16 melanoma (40,41). These compounds are difficult to synthesize and are quite unstable making them unlikely candidates for therapeutic agents.

Phenolic melanin precursors, that may structurally resemble the natural substrate tyrosine, provide a rational approach to the design of antitumor agents for melanoma. Many previous attempts at developing chemotherapeutic agents against melanoma have been based upon dopa and related catecholic compounds that may cause general cytotoxicity from auto-oxidation (26,33,34). Alternatively phenolic compounds, such as 4-S-CP and 4-S-CAP, have been found to be more potent melanocytotoxic agents than catechols using an in vivo assay system (31). Another phenolic compound, 4-hydroxyanisole (4-OHA), has also been shown to have cytotoxic effects on melanocytes

and melanoma cells, but its toxicity is probably due to oxygen radicals formed extracellularly (42).

If analogs of tyrosine could be taken up by melanoma cells and oxidized intracellularly to cytotoxic quinones, then these neoplastic cells could be selectively killed while sparing normal tissues that do not have tyrosinase. The conversion of these phenolic compounds to toxic intermediates should also be enhanced by increasing the tyrosinase activity of the cell. Tyrosinase can be activated in response to elevated levels of cAMP in a reaction apparently mediated by cAMP-dependent protein kinases (39). Increases in intracellular cAMP levels can be achieved through the administration of the hormone MSH. Cells that had an artificially high tyrosinase activity that resulted from exposure to MSH were much more susceptible to the toxicity of melanin precursors (39). On the other hand, inhibition of tyrosinase should result in a decrease in toxicity. Treatment of cells with phenylthiourea (PTU), a potent inhibitor of tyrosinase, provided complete protection from the toxicity of melanin precursors (39). Cells that do not have the enzyme tyrosinase also show a much lower level of toxicity from these precursors indicating a selectivity for melanin synthesizing cells. A question still remains as to how these melanin precursors might be

incorporated into melanoma cells where they can be metabolized into their cytotoxic derivatives.

1.7 GENERAL INTRODUCTION TO AMINO ACID TRANSPORT

Amino acids are the building blocks for all cellular proteins and can be used for energy metabolism. The requirements of the cell for these amino acids are met from two sources: a) extracellular amino acids taken up into the cells and, b) intracellular biosynthesis of certain of the amino acids. In order to obtain the extracellular amino acids, several transport systems exist in animal cells (Table 1.1) (43-46). Almost all cells possess these membrane transport systems for amino acids in order to accommodate a diverse range of intracellular biochemical requirements. The presence and activity of these transporters can be affected by many factors that influence the growth and differentiation of the particular cell type involved (47).

Studies on the transport of nutrients into tissue culture cells have increased since it was proposed that growth rate and malignancy of tumor cells may, to some extent, be controlled by the transport activity of cell membranes (48). A study of the existing transport systems is a necessary first step in examining the regulation of amino acid transport and in determining to what extent this can affect or be affected by cellular

function. In melanoma cells it has been suggested that the increase in melanin synthesis during the melanotic stage is associated with increased activity of amino acid transport as well as increased tyrosinase activity (49).

As a result of transport studies in various cell types and for a variety of amino acids and analogs, a classification system has been developed with which to compare and categorize these transport systems (Table 1.1) (43-46). These amino acid transport systems have been characterized by two principal methods: a) the study of mutants with transport deficiencies, and b) direct kinetic analysis of amino acid fluxes. In mammalian cells the direct analysis approach has been favored, although difficulties can arise when using transported substrates as inhibitors or when different transport systems show overlapping specificities for particular amino acids (32).

In general, transport is dependent on many factors including cell type, proliferation rate, general metabolic activity, and cell specific metabolic activity (44,47). Therefore, it is important to strictly control the conditions under which amino acid transport will be studied in order to clearly interpret the results. The utilization of an <u>in vitro</u> model has made this much simpler since all of the parameters for cell growth can be carefully controlled.

Neutral amino acids such as tyrosine, tryptophan, phenylalanine, alanine and leucine have had their transport mechanism studied in many different cell types (47,50-59). These transport systems have been grouped according to various parameters including substrate specificity and sodium ion dependence (Table 1.1). Three ubiquitous transport systems for neutral amino acids have been characterized; L, A, ASC, as well as the cell specific transport systems T, $B^{o,+}$, $b^{o,+}$ and asc.

System L is a widely distributed transport system that is capable of transporting many different amino acids with different structures. This Na^+-independent transport system was first described for the transport of leucine, but is also capable of accommodating amino acids containing aromatic rings (50,60). System L has a characteristic substrate, 2-aminobicyclo-(2.2.1)-heptane-2-carboxylic acid (BCH), that has been found to be specific for only this transport system. This can be very useful when trying to elucidate the mechanism of transport of a compound.

A variant of the L transport system has been described for mammalian red blood cells (61,62) and rat hepatocytes (63). System T is specific for transporting neutral amino acids that possess aromatic rings such as tyrosine, tryptophan and phenylalanine (61). It has been characterized as a Na^+-independent transport system, with

tryptophan serving as its model substrate. In all cell types investigated except mammalian red blood cells and rat hepatocytes, the substrate specificity of system T completely overlaps that of system L resulting in the identification of only a single transport system for the larger neutral amino acids.

Amino acids with shorter side chains have been found to be transported by system A or ASC. Much study has been done on system A (alanine-prefering) transport since it has been discovered to be important in the liver for the alanine-glucose cycle (45) and identified as a possible target for oncogenic action in neoplastic cells (64). System A is most effective for small neutral amino acids such as alanine, glycine, serine and the analog 2-aminoisobutyric acid (AIB) (44,48). This transport system is Na^+-dependent, has as its model substrate 2-methylaminoisobutyric acid (MeAIB), and is sensitive to hormonal stimulation (48).

A second Na^+-dependent neutral amino acid transport system has been identified. This system which primarily transports alanine, serine, and cysteine is designated ASC. System ASC has a narrower range than system A as to the length of the amino acid side chains it finds acceptable (45,65). In addition, system ASC can be distinguished from system A on the basis of its intolerance of N-methylated substrates (eg. MeAIB) (44).

A Na^+-independent version of this transporter has been characterized in human red blood cells and has been designated system asc (62). With the exception of the Na^+-dependence, both ASC and asc have similar substrate characteristics and kinetics.

More recently, Van Winkle et al (58,59) have described the presence of two additional processes that are capable of transporting bulky zwitterionic or cationic amino acids. $B^{o,+}$ designates the Na^+-dependent form, and $b^{o,+}$ the Na^+-independent. So far, these transport mechanisms have only been identified in the preimplantation mouse conceptuses model.

1.8 AMINO ACID TRANSPORT AND TOXICITY

The relationship between cellular transport and cytotoxicity has been studied for various chemotherapeutic agents. One amino acid analog of interest, the L-phenylalanine mustard melphalan, is used clinically in the treatment of multiple myeloma (66) and malignant melanoma (67). The transport of this compound into various cell types has been studied and its effects as a chemotherapeutic agent have been linked, at least partially, to its transport (66,68,69).

The mechanism of transport of melphalan has been found to be cell-type dependent. When investigating L5178Y murine leukemia lymphoblasts, melphalan uptake was

found by Begleiter et al (66) to be strongly inhibited by the leucine analog BCH, but not by the System A specific substrate MeAIB or the System ASC substrate AIB. The transport was not affected by the addition of alkylating agents, while amino acid analogs caused a decrease in the melphalan transport (64). Therefore, melphalan transport closely resembles amino acid transport, potentially useful information about its interaction with the cells.

In other murine leukemia cells melphalan appears to be transported by two separate neutral amino acid transport systems, one of which is a BCH sensitive, sodium-independent L system and the other a BCH sensitive, sodium dependent system (68).

Melphalan is transported by the murine leukemia L1210 cell through carriers of the L-type (69). The reduction in cytotoxicity by single amino acids correlated with a reduction in drug uptake. Additional studies indicated that melphalan transport is mediated through a similar system by cells maintained in vivo and that administration of leucine with melphalan negated the latter's therapeutic efficacy. Studying the cytotoxic effects in relation to the inhibition of amino acid transport allowed for a partial explanation of the action of this compound.

With melanoma cells the uptake of a tyrosine homolog, 4-S-CP has been partially studied (Figure 1.3)

(26,70). 4-S-CP was found to be specifically incorporated into the melanotic melanoma cells (HMV II) which are engaged in active melanin synthesis, but was scarcely incorporated into HeLa S3 or HMV-I cells which have no activity for melanin synthesis (70). In this system, active tyrosinase is required for the incorporation of 4-S-CP. There is evidence that 4-S-CP and related compounds are effective against malignant melanoma (26-33). Therefore, there may exist a relationship between the incorporation of these compounds into melanoma cells and the observed cytotoxicity similar to that found for the amino acid analog melphalan. However, it is still unclear how and why tyrosine and the tyrosine homolog 4-S-CP are selectively transported into melanoma cells. This study characterizes the transport of tyrosine, <u>in vitro</u>, for the human melanotic melanoma cell line, SK-MEL 23.

Table 1.1

General amino acid transport systems in animal cells

Na^+-dependent	Substrates
ASC	alanine, serine, cysteine, AIB
GLY	glycine, serine, sarcosine
N	glutamine, asparagine
A	alanine, glycine, proline, AIB, MeAIB, methionine
$B^{o,+}$	cationic, bulky amino acids

Na^+-independent	Substrates
L	leucine, phenylalanine, alanine, valine, tyrosine, tryptophan, BCH
T	tryptophan, tyrosine, phenylalanine
asc	alanine, serine, cysteine, AIB
C	alanine, cysteine
$b^{o,+}$	cationic, bulky amino acids

Cationic	Substrates
y^+ (Na^+-independent)	diamino acids, arginine, cationic form of histidine

Anionic	Substrates
X^- (Na^+-dependent)	aspartate, glutamate
x^- (Na^+-independent)	aspartate, glutamate

Figure 1.1

Melanocyte structure

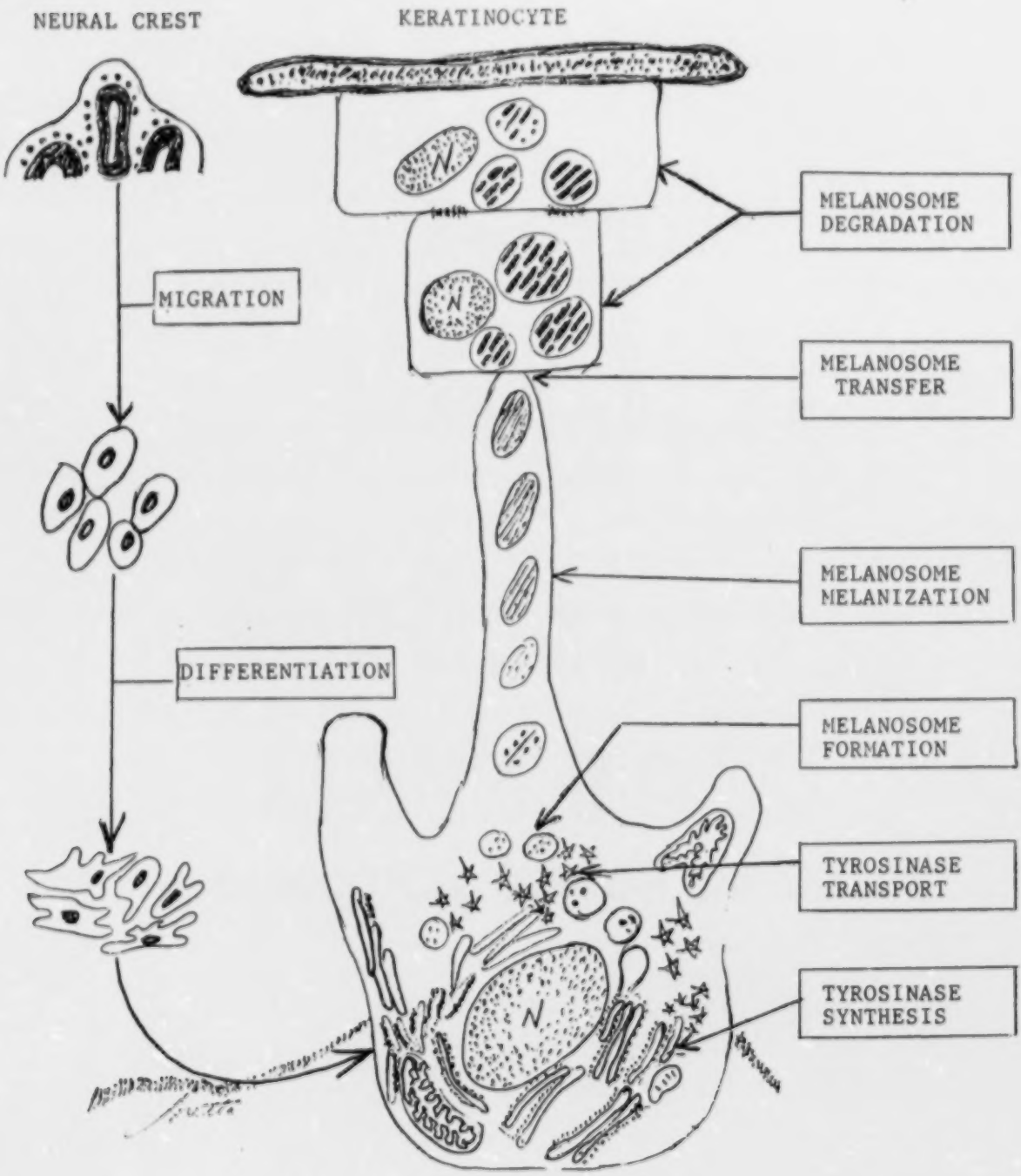


Figure 1.2

Melanin synthetic pathway

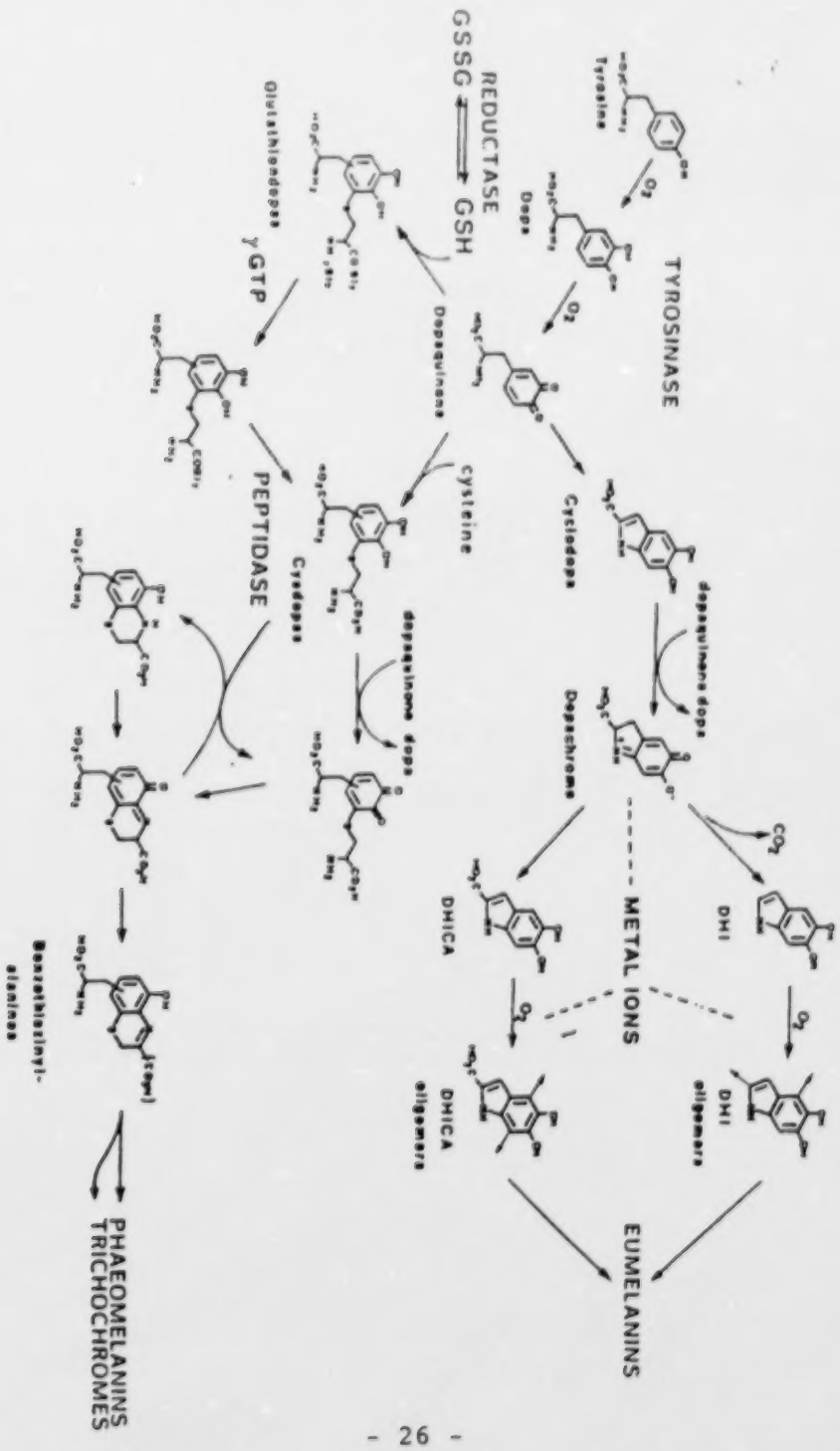
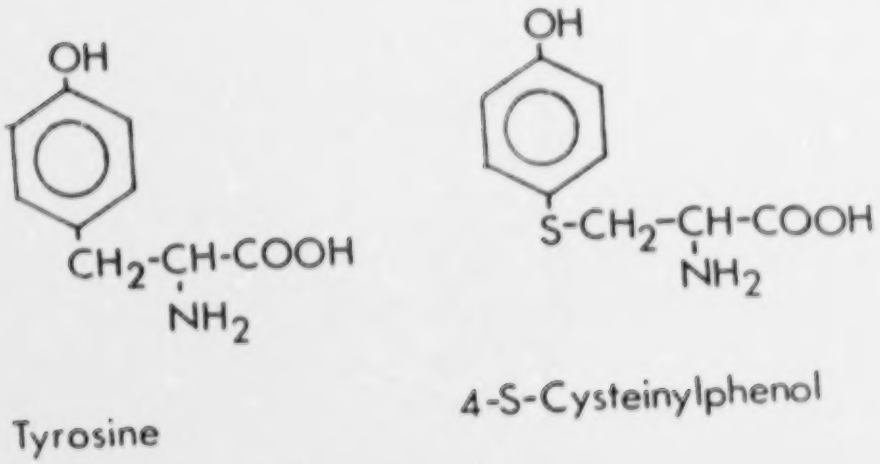


Figure 1.3

Chemical structure of the amino acid tyrosine and its sulfur homolog 4-S-cysteinylphenol (4-S-CP)



Tyrosine

4-S-Cysteinylphenol

CHAPTER 2
MATERIALS AND METHODS

2.1 MATERIALS

Minimal essential medium (MEM), fetal bovine serum (FBS), trypsin-EDTA, and penicillin-streptomycin were purchased from Gibco (Grand Island, NY). The 24-well plates and T-150 flasks used for culturing the cells were obtained from Corning (Corning, NY). L-[ring-3,5-^{3}H]-tyrosine was obtained from NEN (Mississauga, Ontario) at a specific activity of 46.7 Ci/mmol and stored at 4° C. The system L specific substrate, BCH, was obtained from Calbiochem (San Diego, CA). The other inhibitors and chemicals used were of the highest quality available and were obtained from Sigma Chemical Co. (St. Louis, MO).

4-S-CP was synthesized by Dr. S. Ito (Fujita Gakuen University, Toyoake, Japan), the synthesis being reported previously (27,71). Briefly, a mixture of phenol and L-cystine was refluxed in HBr for 2 hours. The resulting orange solution was evaporated to dryness and the residue dissolved in water. The acidity of the solution was adjusted to pH 5 and allowed to stand at 4° C for one week. Colorless 4-S-CP precipitated and was collected.

2.2 EQUIPMENT

Liquid scintillation counting was performed using the xylene based scintillation cocktail Aquasol-2 (NEN, Mississauga, Ontario) in a Beckman LS 3801 liquid scintillation counter. For counting the tritiated tyrosine on this equipment, the following settings were used: 10 minute counts, Beckman external standard method of quench correction (H#), a channel setting of 0-400, and automatic quench compensation to maximize the counting efficiency. Results were programmed to calculate disintegrations per minute (dpm) based on a quench correction curve and converted to nmol tyrosine using equation 2.1.

Equation 2.1

$$(\text{nmol tyrosine})_i = \frac{(\text{nmol tyrosine})_t (\text{sample dpm} - \text{blank dpm})}{(\text{total dpm} - \text{blank dpm})}$$

Where;

$(\text{nmol tyrosine})_i$ = represents the internalized amount of tyrosine

$(\text{nmol tyrosine})_t$ = represents the total tyrosine in the uptake medium

sample dpm = the disintegrations per minute from the experimental sample

blank dpm = the disintegrations per minute from the wells subjected to identical experimental conditions, but in the absence of cells

total dpm = the disintegrations per minute in the uptake medium used in the experiment

2.3 PROTEIN ASSAY

The protein content was determined using the Bradford dye-binding assay available in kit form from Bio-Rad (72). This assay relies on the differential color change of the assay dye, detectable at 595 nm, in the presence of various concentrations of protein.

The assay involved the mixing of 0.1 mL of sample or standard with 5 mL of 1:4 diluted dye reagent. The solution was gently mixed and after a period of five minutes to one hour, the absorbance at 595 nm was determined. The reagent blank consisted of sample solution with no protein and protein dye.

The color change was detected on a Beckman DU-65 spectrophotometer and then compared against a standard curve prepared using bovine serum albumin (BSA) as the standard (Figure 2.1).

2.4 CELL CULTURE

The human melanotic melanoma cell line, SK-MEL 23, was the subject of investigation in these studies. This cell line was kindly supplied by Dr. A. N. Houghton of the Memorial Sloan-Kettering Cancer Centre (NY). Originally, this adherent cell line was derived from a metastatic lesion of human melanoma and has been characterized as having both a high tyrosinase level and being highly pigmented (73).

A frozen stock of SK-MEL 23 cells kept in liquid nitrogen and aliquots were defrosted and grown when required. Cells were tested for the characteristic high tyrosinase activity before use.

These cells were cultured in a Steri-cult 200 incubator (Forma Scientific) under the following standard conditions: 37° C, 5% CO_2, and 95% humidity. The culture medium was MEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 ug/mL streptomycin. After 2-3 days of growth in 150 cm^2 tissue culture flasks, the subconfluent cells were collected by trypsinization and transferred to 24 well plates where the transport assays were performed.

2.5 TYROSINASE ASSAY

One characteristic that is unique to this cell line is the tyrosinase activity. Each frozen stock was thawed and assayed for tyrosinase activity before use in the transport studies.

The tyrosinase assay, as modified by Townsend et al (74), is based upon the enzymatic conversion of L-[3,5-^{3}H]tyrosine to dopa with the release of ^3H_2O in the process.

The assay is initiated by the addition of reaction mixture consisting of 21,000 dpm L-[3,5-^{3}H]tyrosine, 1.5 mM tyrosine, 0.6 mM dopa, TES/TRIS pH adjusting buffer

(pH 7.8) and 0.025 mL sample. The tubes are incubated at 37°C for 3 hours and the reaction stopped by the addition of 0.5 mL of 1% charcoal. The tubes are mixed and incubated at 0°C for 15 minutes and centrifuged at 1000g for 10 minutes. A 0.4 mL aliquot is transferred to a Dowex 50Wx12 200-400 mesh column. The column is then washed with 0.8 mL of 0.1 N citric acid. The eluent is collected for liquid scintillation counting and the tyrosinase activity determined based upon the ^3H_2O radioactive counts.

The tyrosinase activity varies with the time of incubation (Table 2.1). The highest levels were observed 1 or 2 days after thawing and incubating under the standard cell culture conditions. Beyond two days the tyrosinase activity decreases. This decrease is probably due to the increase in the number of cells in the culture flasks. Once the cells are sub-cultured, the level of tyrosinase activity returns to the previously high level.

2.6 MEASUREMENT OF TYROSINE TRANSPORT

Tyrosine transport was measured using a monolayer-cell washing technique (75,76). To obtain the cell monolayer, SK-MEL 23 cells were cultured in 24 well plates under the standard condition. Initially, the cells were seeded at a density of 4 X 10^4 per well in MEM medium and allowed to grow for 2-3 days prior to the

assay procedure being performed. These conditions resulted in 70-80% confluence for the assay procedure.

To prepare the cells for the assay procedure, the monolayer was washed 3 times with a transport buffer (pH 7.4) consisting of 140 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.9 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 25 mM HEPES (328 mOs). The cells were then preincubated in this transport buffer for 30 minutes prior to the initiation of the transport assay.

The transport assay was initiated by the addition of the uptake medium made by supplementing the transport buffer with 1 uCi/mL L-[ring-3,5-^{3}H]-tyrosine, unlabeled tyrosine to the desired concentration and, when appropriate, with unlabeled inhibitors. The cells were allowed to incubate with the uptake medium at room temperature for specific periods of time depending on the experimental conditions. Transport was terminated by aspiration of the uptake media and rapidly washing with three 1.0 mL washes of ice-cold transport buffer. The total wash sequence was completed in less than 20 seconds. One mL of 0.2 N NaOH was added to each well and allowed to stand overnight to dissolve the cells. From this a 0.1 mL sample was taken to determine radioactivity by liquid scintillation counting. A second sample was taken and assayed for protein content by the Bradford dye-binding method using BSA as the standard (72).

Table 2.1

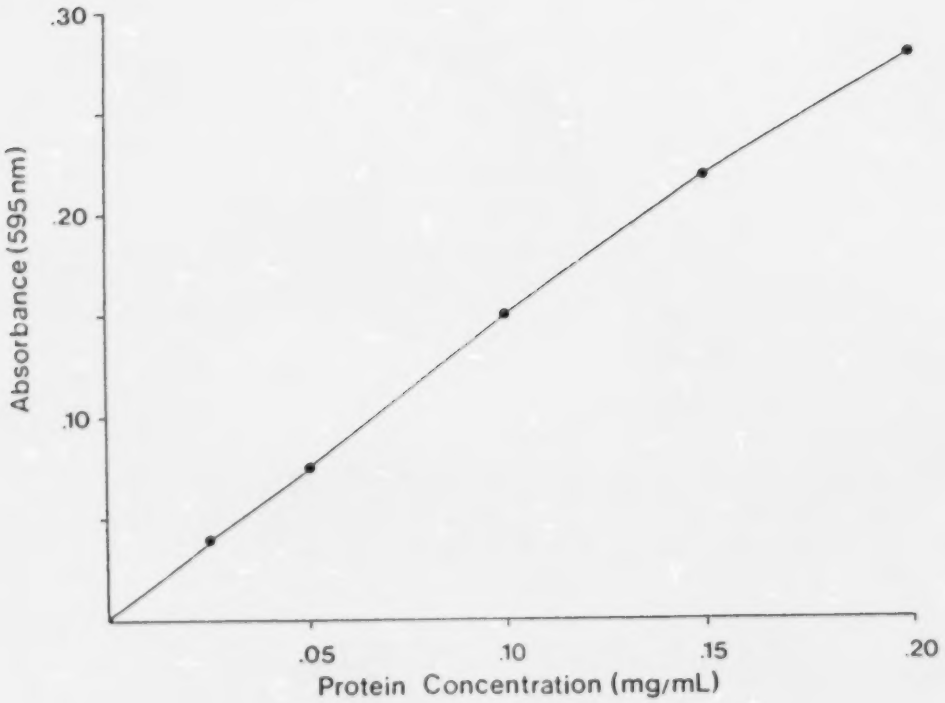
Tyrosinase activity of SK-MEL 23 cultured cells

	Tyrosinase activity (nmol/hr/10^6 cells)
Day 1	4.1 ± 0.1
Day 2	5.2 ± 0.4
Day 3	2.8 ± 0.2
Day 4	0.2 ± 0.01

Tyrosinase activity was determined for cultured SK-MEL 23 cells under standard cell culture conditions after thawing frozen stocks. L-[3,5-^{3}H]tyrosine is enzymatically converted to dopa and ^3H_2O, which is then counted by a liquid scintillation counter. Values are means ± SE of triplicate determinations.

Figure 2.1

Typical standard curve used in the determination of protein content by the Bradford assay with bovine serum albumin (BSA) as the standard.



The Bradford assay for protein determination relies on the detection of a color change at 595 nm in response to the addition of protein to the assay dye. The protein assay consisted of 0.1 mL of sample or standard added to 5 mL of 1:4 diluted dye reagent. After mixing, the solution was allowed to stand for five minutes to one hour. This color change is detected on a spectrophotometer and compared to the standard curve to obtain the experimental value. Values are means ± SE of triplicate determinations. The lack of error bars represents errors so small that they fall within the points.

CHAPTER 3
RESULTS

3.1 TIME COURSE OF TYROSINE TRANSPORT

The SK-MEL 23 human melanoma cells were washed and preincubated in the amino acid free transport buffer for 30 minutes prior to the initiation of the transport assay. This would reduce the influence of intracellular stores of amino acids on the transport of tyrosine (75,76). Following the preincubation period, a time course of tyrosine uptake was followed by incubating the cells for up to 60 seconds in an uptake medium containing ^{3}H-tyrosine and a final tyrosine concentration of 0.1 mM. The amount of tyrosine taken up into the cells was determined using a liquid scintillation counter and Equation 2.1. The amount of protein present per well was determined using the Bradford assay. The uptake can be seen to reach a plateau at 60 seconds (Figure 3.1). There was no difference when Na^{+} was removed from the transport buffer and replaced with K^{+}. All subsequent experiments used an incubation time of 20 seconds to approximate initial rates of transport.

3.2 KINETICS OF TYROSINE TRANSPORT

Kinetics is the study of the rates of movement - in the present context, the rate of movement of a transported substrate across a biological membrane. To understand the kinetics of transport is an attempt to understand the interaction between the substrate and the transporter. Two common values used to describe this interaction are the K_m and V_{max} values. K_m is the kinetic constant that represents the affinity of the substrate for the transporter expressed in concentration units. The K_m is the concentration at half maximal transport velocity. There exists an inverse relationship between the K_m value and substrate affinity, that is the lower the K_m value, the higher the substrate-transporter affinity.

The V_{max} value is the maximal velocity of the transport expressed as units of substrate per unit time period per unit of cell number. The V_{max} occurs when the transport system is saturated with substrate. Under constant cell culture conditions, the K_m and V_{max} values should remain relatively constant.

When the conditions of cell growth are altered, the K_m and V_{max} values may change depending on the cellular response to the change. There are four possible scenarios for the transporters in response to these changes: 1) K_m constant, V_{max} constant, 2) K_m changes,

V_{max} constant, 3) K_m constant, V_{max} changes, and 4) K_m changes, V_{max} changes. K_m values will vary if the nature or type of transporter is altered in the response. In this case V_{max} values may or may not change. If the V_{max} value changes, but the K_m remains constant, this indicates a change in the number of transporters involved, or an increase in the turnover of the transporter.

Transport kinetics are studied by observing the transport characteristics at various substrate concentrations. The relationship between substrate concentration and transport velocity can then be interpreted using various mathematical or graphical methods to obtain the values of interest.

The Michaelis-Menton equation also known as the basic velocity equation or ligand binding equation has many useful interpretations in the fields of enzyme and transport kinetics (77,78).

Equation 3.1

Michaelis-Menton Equation

$$V = V_{max} \frac{[S]}{[S] + K_m}$$

Where;

V = rate of transport of species into the cell

[S] = concentration of species outside of the cell

K_m = concentration at half maximal transport across the membrane

V_{max} = the rate of transport if the transport system is saturated with substrate

Since a plot of the Michaelis-Menton equation (V vs [S]) yields a hyperbola, it is difficult to determine V_{max} and the substrate concentration that yields half of the V_{max} value ie., K_m.

To facilitate the determination of the kinetic constants, the data are usually plotted in a linear form. The Lineweaver-Burk reciprocal plot is the most widely used diagnostic plot.

Equation 3.2

Lineweaver-Burk Equation

$$\frac{1}{V} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}$$

Where;

V = rate of transport of the species into the cell

[S] = concentration of species outside the cell

K_m = concentration at half maximal transport across the membrane

V_{max} = the rate of transport if the transport system is saturated with substrate

The plot of 1/V vs 1/[S] is linear with;

slope = K_m/V_{max}

y-intercept = $1/V_{max}$

x-intercept = $-1/K_m$

Care must be taken to choose substrate concentrations in the neighborhood of the K_m value otherwise the graph will be too horizontal or too vertical to allow for proper interpretation of the intercepts. The use of the Lineweaver-Burk plot in the evaluation of kinetic data has been criticized for two major reasons: 1) equal increments of [S] that yield equally spaced points on the basis of the V vs [S] plot do not yield equally spaced points on the reciprocal plot and more importantly, 2) small errors in the determination of V are magnified when reciprocals are taken. This means that one or two "bad" points can introduce considerable error to the slope of this plot.

Other linear transformations of the basic equation have been derived and under certain circumstances may be more suitable and yield more reliable estimates of the kinetic constants.

The Woolf-Augustinsson-Hofstee plot of V vs V/[S] does not involve reciprocals and as a result may be more reliable when the error in V is significant.

Equation 3.3

Woolf-Augustinsson-Hofstee Equation

$$V = -K_m \frac{V}{[S]} + V_{max}$$

Where;

K_m = concentration at half maximal transport across the membrane

V = rate of transport of the species into the cell

[S] = concentration of species outside of the cell

V_{max} = the rate of transport if the transport system is saturated with substrate

The plot of V vs V/[S] is linear with;

slope = $-K_m$

y-intercept = V_{max}

x-intercept = V_{max}/K_m

Since no reciprocals are required, this eliminates the errors that occur in their determination.

The kinetics of tyrosine transport in the SK-MEL 23 cell line was studied using the monolayer cell washing technique at tyrosine concentrations ranging from 0.05-0.8 mM. The tyrosine uptake was determined by liquid scintillation counting and protein content by the Bradford assay. When the transport velocity at each of these concentrations is graphed as a function of tyrosine concentration, a typical saturation curve is produced (Figure 3.2).

Mathematical manipulation of the basic equation allows us to use either the Lineweaver-Burk plot of 1/V vs 1/[S] (Figure 3.3), or the Woolf-Augustinsson-Hofstee plot of V vs V/[S] (Figure 3.4) to graphically determine the K_m and V_{max} values for tyrosine transport. When

graphed, both of these linear transformations of the Michaelis-Menton equation resulted in the determination of similar K_m and V_{max} values. For the Lineweaver-Burk plot, the K_m is the negative inverse of the X-intercept and the V_{max} the inverse of the Y-intercept (K_m = 0.164 mM, V_{max} = 21.4 nmol/min/mg protein). Because of the potential problems with the error when performing the Lineweaver-Burk transformation, the Woolf-Augustinsson-Hofstee plot, considered more reliable, was used for the final determination of the kinetic parameters. From this linear graph the K_m is represented by the negative slope, and the V_{max} by the y-intercept. Based on the best fit line determined statistically using the SPSS linear regression program, the K_m was calculated to be 0.164 $\pm$ 0.016 mM and the V_{max} to be 21.6 $\pm$ 1.1 nmol/min/mg protein (mean SE, n=3).

3.3 INHIBITION ANALYSIS

The use of inhibitors can be very useful in identifying the nature of amino acid transport. Certain amino acids and their analogs have been shown to be specific for one of the previously described transport systems (Table 1.1). The nomenclature used to describe the different systems reflects the specific nature of some of these compounds (eg. system L for leucine, system

T for tryptophan, and system ASC for alanine, serine, and cysteine).

If any of these specific substrates can be shown to inhibit the transport of tyrosine into the cells, then this would be a good indication that similar transport mechanisms are involved. By using a number of different inhibitors, the nature of the transport of the substrate in question can be determined.

Using the monolayer-cell wash procedure, the inhibition of tyrosine transport into SK-MEL 23 cells was studied in the presence of various amino acids and their analogs. Inhibition analysis was performed using a tyrosine concentration of 0.1 mM and an inhibitor concentration of 1.0 mM in the uptake medium. The transport of radiolabeled tyrosine was determined by liquid scintillation counting and protein content by the Bradford assay.

Of the compounds tested as tyrosine transport inhibitors, the most significant inhibition came from the aromatic amino acids, tryptophan (78.1%) and phenylalanine (77.1%), and from the branched side chain amino acid leucine (66.6%) (Table 3.1). The leucine analog, BCH, a specific substrate of system L transporter, and the sulfur homolog of tyrosine, 4-S-CP, were also very effective inhibitors of tyrosine transport with percent inhibition being 63.0% and 61.2%

respectively. The remaining compounds had little or no effect on the transport of tyrosine in this cell line with percent inhibition being less than 30% in each case. The absence of substantial inhibition with the cationic amino acid arginine (14.3%) rules out any involvement of the recently described neutral and cationic amino acid transporters $B^{0,+}$ and $b^{0,+}$.

These experiments were repeated in the absence of Na^+ in the uptake medium by substituting KCl for NaCl producing a K^+ medium. The inhibitors that produced the greatest inhibition in the presence of Na^+, also showed the greatest inhibition in the K^+ medium. Those values did not differ substantially between the two groups.

The inhibitors that were in the group with less than 30% inhibition in the presence of Na^+ again accounted for less than 30% inhibition in the K^+ medium.

3.4 SODIUM DEPENDENCE OF TYROSINE TRANSPORT

There are two major categories of amino acid transport systems that have been identified, Na^+-dependent and Na^+-independent (Table 1.1). To directly compare the transport system for tyrosine in SK-MEL 23 human melanoma cells in the presence or absence of Na^+, the transport was studied at two different tyrosine concentrations. The Na^+ was replaced in the K^+ uptake medium by substituting KCl for NaCl. The monolayer-cell

wash technique was used with an incubation time of 20 seconds to approximate the initial rate of transport. Tyrosine uptake was determined by liquid scintillation counting and protein content by the Bradford assay. At both the 0.1 mM and 0.6 mM tyrosine concentrations, there was no statistical difference in the observed transport (Table 3.2). This result is in agreement with the time course and inhibition analysis where the presence or absence of Na^+ in the uptake medium had no measurable effect.

3.5 SEQUENTIAL INHIBITOR ANALYSIS

From the initial inhibition analysis, it is apparent that there are several amino acids and their analogs that are very good inhibitors of tyrosine transport. These include phenylalanine, tryptophan, leucine, the leucine analog BCH, and the S-homolog of tyrosine, 4-S-CP. Of these effective inhibitors, tryptophan and BCH were chosen for further analysis because each has been associated with a specific amino acid transport system in other cell types (48,61,62). Tryptophan is the characteristic substrate for system T and BCH the specific substrate for system L. The tyrosine homolog, 4-S-CP, was also further analyzed because of its close structural similarity to tyrosine (Figure 1.3) and its relatively high inhibition of tyrosine transport (61.2%).

First, tryptophan or BCH was added alone to 0.1 mM tyrosine uptake medium at concentrations ranging from 0-9 mM. The uptake media was incubated with SK-MEL 23 cells for 20 seconds, and using the monolayer-cell wash procedure, the tyrosine transport was determined. The tyrosine uptake was determined by liquid scintillation counting and protein content by the Bradford assay. Transport inhibition was expressed as a percentage of control transport in which cells were incubated with only the tyrosine added to the uptake medium. Both tryptophan and BCH showed a similar effect due to increasing inhibitor concentration on tyrosine transport inhibition (Figure 3.5). There was a rapid increase in the inhibition caused by increasing the concentration of these compounds followed by a plateau phase in which there was no further inhibition observed.

Next, these two inhibitors, along with the synthetic tyrosine homolog, 4-S-CP, were sequentially added at increasing concentrations and the inhibition of tyrosine transport was followed in a similar fashion. Sequential addition of these tyrosine transport inhibitors would allow for the identification of a transport system or systems that may be masked by the presence of a higher capacity transporter or a transporter with overlapping specificities. The SK-MEL 23 cells were incubated with 0.1 mM tyrosine and either: a) increasing concentrations

of BCH, 2.25 mM BCH plus increasing concentrations of 4-S-CP, and 2.25 mM BCH plus 10 mM 4-S-CP plus increasing concentrations of tryptophan added simultaneously, or b) the addition of the same compounds at the same concentrations but reversing the order of addition of BCH and tryptophan (Figure 3.6). Tyrosine uptake was determined by liquid scintillation counting and protein content by the Bradford assay.

The inhibition of tyrosine transport in response to the increasing concentrations of these compounds was similar even when the sequence of addition of the BCH and tryptophan was reversed. That is, there was no further inhibition over and above that which was seen with the single inhibitor alone upon addition of the second or even third inhibitor. Since each of these compounds alone can cause substantial inhibition of tyrosine transport, this would indicate that all of the inhibitors were working in a similar manner to inhibit tyrosine transport.

3.6 DIXON PLOT FOR 4-S-CP

4-S-CP is structurally related to tyrosine (Figure 1.3) and the potential chemotherapeutic effects are of interest. In order to more closely determine the nature of the 4-S-CP inhibition of tyrosine transport, the

inhibition constant (K_i) for 4-S-CP inhibition was determined graphically using the Dixon plot (78).

Equation 3.4

Dixon Equation

$$\frac{1}{V} = \frac{K_m}{V_{max}\ K_i\ [S]}\ [I] + \frac{1}{V_{max}}\ \left(1 + \frac{K_m}{[S]}\right)$$

Where;

V = rate of transport of species into the cell

K_m = concentration at half maximal transport across the membrane

V_{max} = the rate of transport if the transport system is saturated with substrate

K_i = inhibition constant

[S] = concentration of species outside of the cell

[I] = concentration of inhibitor outside of the cell

By plotting 1/V vs [I] at different fixed concentrations of substrate provides a way of identifying the type of inhibition and of determining the K_i. The lower the value of K_i, the greater is the degree of inhibition at any given substrate and inhibitor concentration. K_i is equivalent to the concentration of inhibitor that doubles the slope of the 1/V vs 1/[S] plot.

Using 0.05, 0.1, and 0.2 mM tyrosine concentrations and increasing concentrations (0.05-1.0 mM) of 4-S-CP in the uptake media, the tyrosine transport was followed

utilizing the monolayer-cell wash procedure with an incubation time of 20 seconds at room temperature. Tyrosine uptake was determined by liquid scintillation counting and protein content by the Bradford assay. The results can be graphically expressed to determine the inhibition constant for the effect of 4-S-CP on tyrosine transport, the K_i value (Figure 3.7). The Dixon plot shows that the 4-S-CP acts as a competitive inhibitor of tyrosine transport and the K_i value was found to be 0.3 mM.

3.7 OSMOLARITY EFFECTS

Cells can regulate their volume by the movements of amino acids across the membrane (79). In vitro, this can be simulated by changing the osmolarity of the incubation solution. By varying the concentration of NaCl and not substituting for the loss of those ions, the effect of changing the osmolarity of the uptake medium on tyrosine transport in SK-MEL 23 melanoma cells could be determined. Using the uptake medium containing 0.1 mM tyrosine and the monolayer-cell wash technique, the NaCl concentration was varied from 70-150 mM without compensation for the change in NaCl concentration producing uptake media with 188-328 mOs. The change in NaCl concentration had no effect until the concentration was decreased to 70 mM (Figure 3.8). Even at this low

NaCl concentration, the tyrosine transport was still 78% of the control value. Above the 140 mM NaCl control, the transport was also unchanged. Therefore, there seems to be little osmolarity effects on tyrosine transport even when the NaCl concentration in the uptake media was decreased by half and the osmolarity of the solution was decreased from 328 to 188 mOs.

3.8 MANIPULATION OF TYROSINE TRANSPORT

Tyrosine is required by SK-MEL 23 melanoma cells for both general protein synthesis and for the production of pigment. Changing the cellular requirements for tyrosine by altering the melanin synthesis pathway may affect the transport of this essential amino acid into the melanoma cells. If a regulation mechanism for the transport of tyrosine and tyrosine homologs could be found, then this may be a mechanism to increase the accumulation of cytotoxic tyrosine homologs in melanoma cells.

There are several factors that could contribute to a change in melanogenesis. One is by decreasing the activity of tyrosinase in the melanosome. Phenylthiourea (PTU) has been shown to inhibit tyrosinase, thereby decreasing or eliminating the activity of this melanin producing enzyme (39,80). Conversely, stimulation of tyrosinase activity can occur in the presence of melanocyte stimulating hormone (MSH). This endogenous

hormone causes an increase in pigment production in human melanoma cells (7,11,39). By incubating the SK-MEL 23 cells with these compounds, we can study the effects of changing melanin synthesis on tyrosine transport.

The SK-MEL 23 cells were seeded into the 24 well plates and allowed to attach overnight. The medium was changed from MEM to MEM supplemented with either 0.1 mM PTU or 2 ug/mL MSH. The cells were then incubated under the standard conditions for two days at which time the tyrosine transport assay was performed. All results were compared to a control group that were grown in the presence of MEM alone. Tyrosine uptake was determined by liquid scintillation counting and protein content by the Bradford assay.

The addition of PTU had no effect on the tyrosine transport in this cell line as the PTU treatment group showed no change from the control group (Table 3.3). On the other hand, stimulation of melanogenesis by the addition of MSH to the growth medium increased the transport of tyrosine by 30% compared to the control group.

Table 3.1

Inhibition of tyrosine transport[a] into malignant melanoma cells *in vitro* by amino acids and amino acid analogs

	% Inhibition	
Inhibitor (1.0 mM)	NaCl medium	KCl medium
tryptophan	78.1 ± 1.0	69.2 ± 1.0
phenylalanine	77.1 ± 1.2	76.1 ± 3.1
leucine	66.6 ± 0.8	55.0 ± 2.6
BCH[b]	63.0 ± 0.8	62.3 ± 1.4
4-S-CP[c]	61.2 ± 2.8	63.0 ± 2.4
cysteine	28.2 ± 0.9	0.0
alanine	18.6 ± 1.1	14.1 ± 1.4
cystine	17.7 ± 1.2	27.9 ± 1.6
arginine	14.2 ± 3.6	0.9 ± 0.9
serine	13.5 ± 0.6	3.5 ± 1.9
glutamine	13.3 ± 2.5	3.4 ± 1.7
MeAIB[d]	9.8 ± 1.9	0.0
AIB[e]	5.8 ± 0.6	0.0

a) Tyrosine transport was measured at a 0.1 mM tyrosine concentration in the presence of 1.0 mM inhibitors. K^+ was used as a replacement for Na^+ in the KCl medium experiments. Cells were incubated at room temperature for 20 seconds in the presence of both tyrosine and the inhibitor. The transport was stopped by aspiration of the medium and washing with ice-cold buffer. Values are means ± SE of triplicate determinations.

b) BCH - 2-aminobicyclo-(2.2.1)-heptane-2-carboxylic acid

c) 4-S-CP - 4-S-cysteinylphenol

d) MeAIB - 2-methylaminoisobutyric acid

e) AIB - 2-aminoisobutyric acid

Table 3.2

Comparison of tyrosine transport into melanoma cells *in vitro* in the presence or absence of Na^+ in the uptake medium

Tyrosine Concentration (mM)	Tyrosine Uptake[a] (nmol/mg protein) NaCl medium	KCl medium	t-test (p-value)[b]
0.1	2.49 ± 0.12	2.87 ± 0.09	$p>0.05$
0.6	6.23 ± 0.81	7.02 ± 0.35	$p>0.2$

a) K^+, in the form of KCl, was used as the replacement for Na^+. Cells were incubated for 20 seconds in transport buffer supplemented with either 0.1 or 0.6 mM tyrosine. The cells were washed 3 times with ice-cold buffer then lysed using 0.2 N NaOH and samples taken for determination of [^{3}H]-tyrosine by liquid scintillation and protein content by the Bradford dye-binding assay. Values are means ± SE of at least triplicate determinations.

b) P-values were obtained using Student's t-test.

Table 3.3

Manipulation of tyrosine transport[a] in SK-MEL 23 cells with PTU and MSH

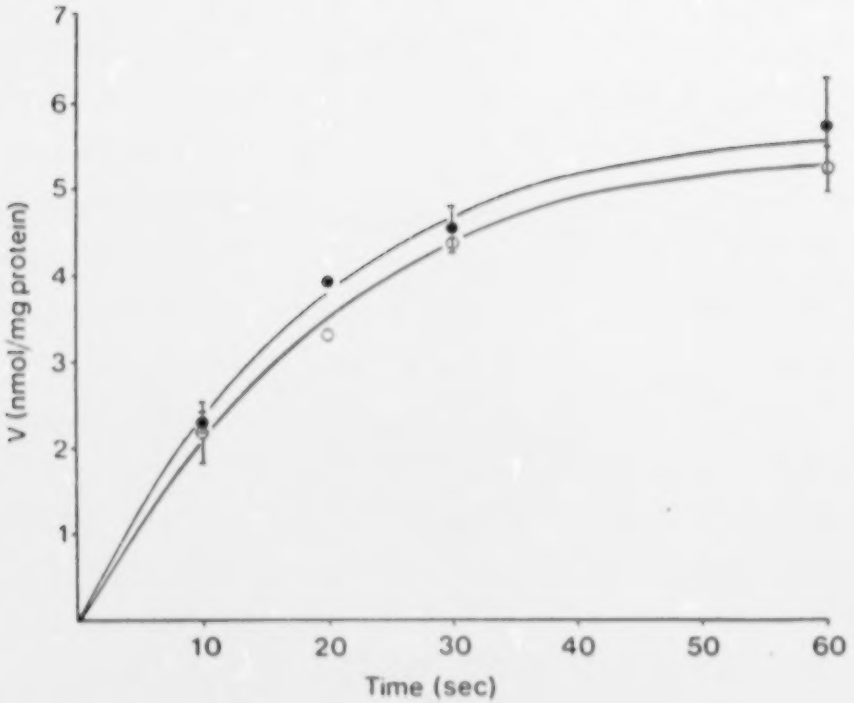
	V (nmol/min/mg protein)	% Control	t-test[b]
Control	6.10 ± 0.02	-	
PTU	6.04 ± 0.44	99	$p>0.5$
MSH	7.92 ± 0.28	130	$p<0.02$

a) Tyrosine transport was measured at a 0.1 mM tyrosine concentration after treatment of the cells for 2 days with either 0.1 mM PTU or 2 ug/mL MSH. Cells were incubated for 20 seconds in the tyrosine supplemented transport buffer. The cells were then washed 3 times with ice-cold buffer then lysed using 0.2 N NaOH and samples taken for determination of [^{3}H]-tyrosine by liquid scintillation and protein content by the Bradford assay. Values are means ± SE of triplicate determinations.

b) P-values were obtained using Student's t-test.

Figure 3.1

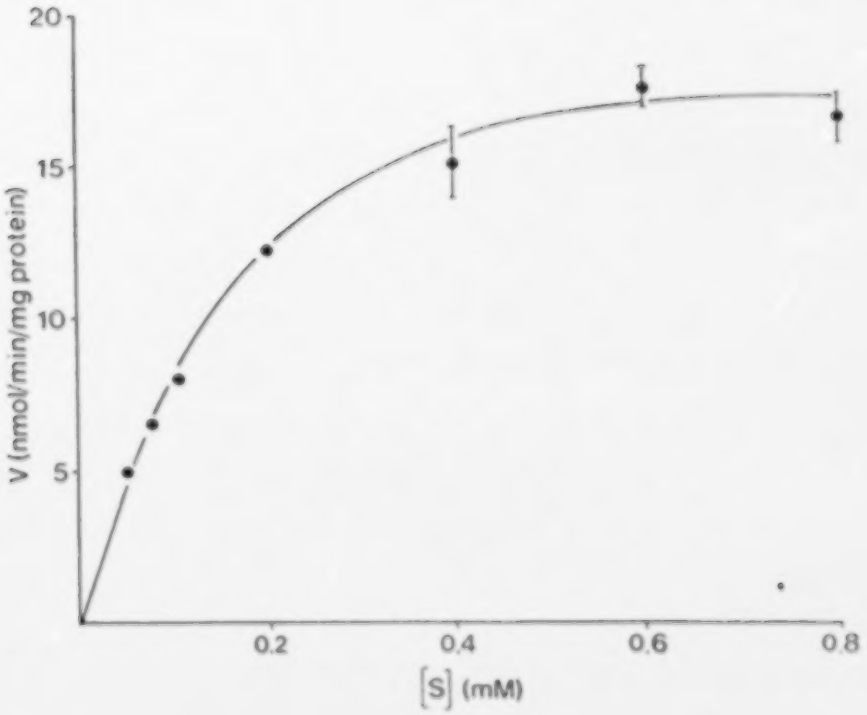
Time course of tyrosine transport in SK-MEL 23 melanoma cells



The time course of tyrosine transport was followed for up to 60 seconds at a tyrosine concentration of 0.1 mM in either NaCl (●) or KCl (○) uptake medium. The cells were then washed 3 times with ice-cold buffer then lysed using 0.2 N NaOH and samples taken for determination of [^{3}H]-tyrosine by liquid scintillation and protein content by the Bradford assay. All subsequent experiments used a 20 second incubation to approximate initial rates of transport. Values are means ± SE of triplicate determinations. The lack of error bars represents errors so small that they fall within the points.

Figure 3.2

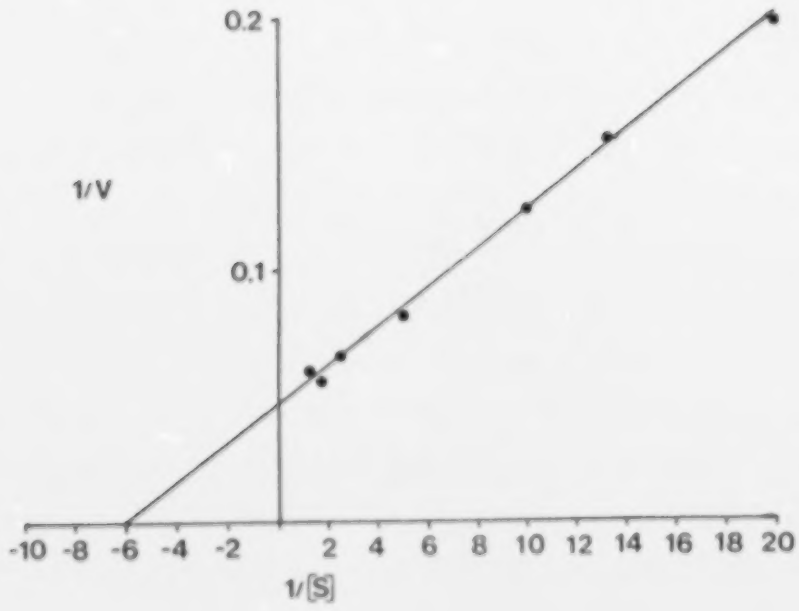
Michaelis-Menton saturation curve of tyrosine transport



Concentration dependence of tyrosine uptake by SK-MEL 23 melanoma cells over the tyrosine concentration range of 0.05-0.8 mM. Transport was measured for 20 seconds in NaCl uptake medium. To stop the transport, cells were washed 3 times with ice-cold buffer and cells lysed using 0.2 N NaOH. Liquid scintillation was used to determine the [^{3}H]-tyrosine uptake and protein content was determined using the Bradford dye binding assay. Values are means ± SE of triplicate determinations. The lack of error bars represents errors so small that they fall within the points.

Figure 3.3

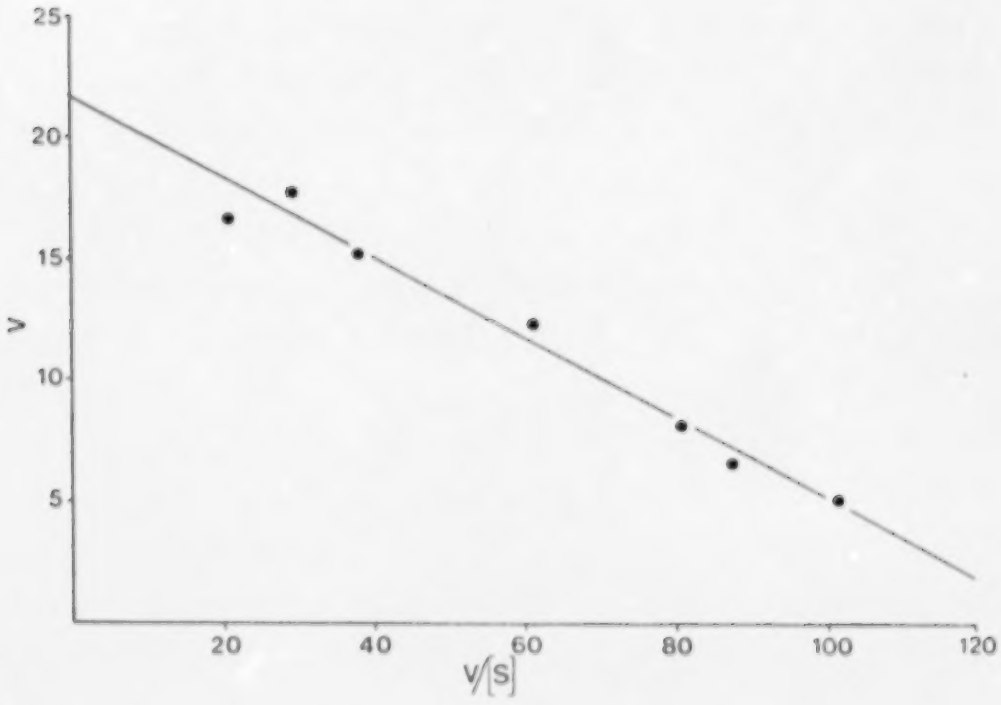
Lineweaver-Burk plot of tyrosine transport data



The Lineweaver-Burk reciprocal plot of 1/V vs 1/[S] can be used to determine K_m and V_{max} values. Tyrosine concentrations from 0.05-0.8 mM were used in the tyrosine transport assay to produce the data for this plot. Cells were incubated for 20 seconds in the tyrosine supplemented transport buffer. The cells were then washed 3 times with ice-cold buffer then lysed using 0.2 N NaOH and samples taken for determination of [^{3}H]-tyrosine by liquid scintillation and protein content by the Bradford assay. Values are means ± SE of triplicate determinations. The lack of error bars represents errors so small that they fall within the points.

Figure 3.4

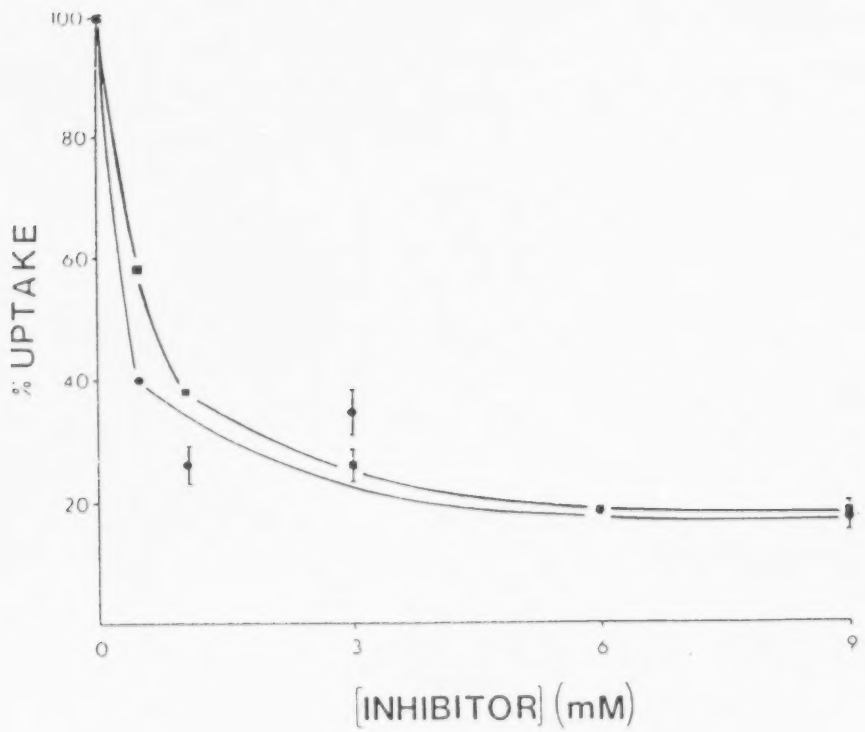
Woolf-Augustinsson-Hofstee plot of tyrosine transport data



Using the Woolf-Augustinsson-Hofstee transformation of the basic Michaelis-Menton equation, a linear relationship is used to determine the K_s and V_{max} values. Tyrosine concentrations ranged from 0.05-0.8 mM and transport was measured for 20 seconds in NaCl uptake medium. The cells were then washed 3 times with ice-cold buffer then lysed using 0.2 N NaOH and samples taken for determination of [^{3}H]-tyrosine by liquid scintillation and protein content by the Bradford assay. Values are means ± SE of triplicate determinations. The best fit line was determined using the SPSS linear regression program. The lack of error bars represents errors so small that they fall within the points.

Figure 3.5

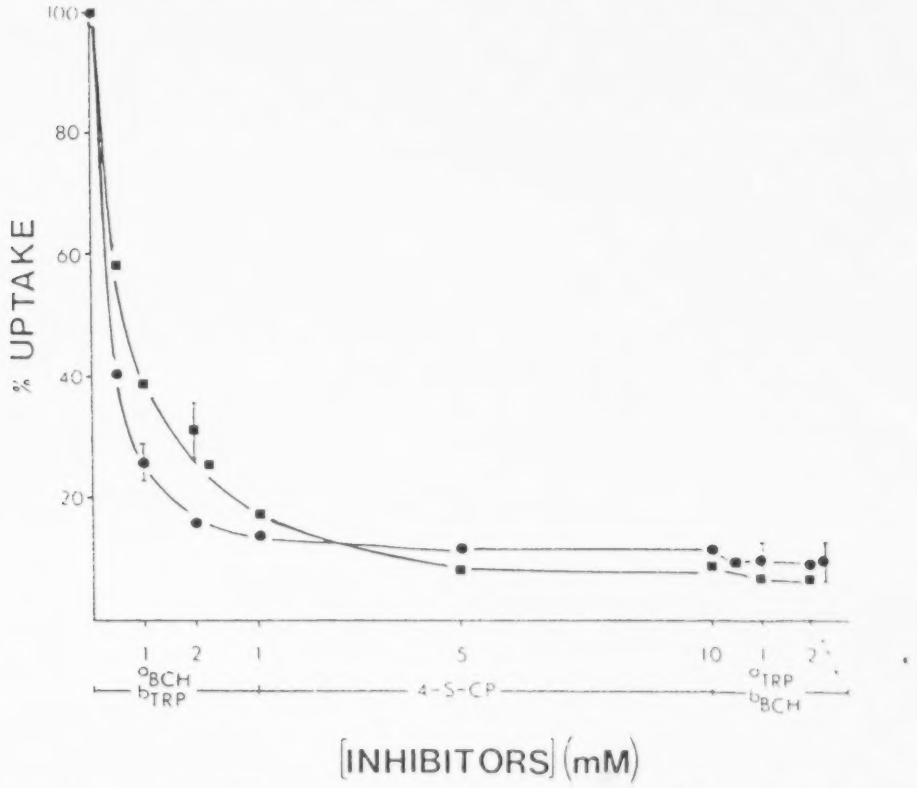
Inhibition of tyrosine transport using either BCH or tryptophan



Tyrosine transport was followed using the NaCl uptake medium supplemented with 0.1 mM tyrosine and 0 - 9 mM BCH or tryptophan as inhibitors. Cells were incubated for 20 seconds in experimental medium. The cells were then washed three times with ice-cold buffer, then lysed using 0.2 N NaOH. Samples were taken for determination of [^{3}H]-tyrosine by liquid scintillation and protein content by the Bradford assay. Control value was 6.82 ± 0.32 nmol/min/mg protein. Values are means ± SE of triplicate determinations. BCH (■); tryptophan (●). The lack of error bars represents errors so small that they fall within the points.

Figure 3.6

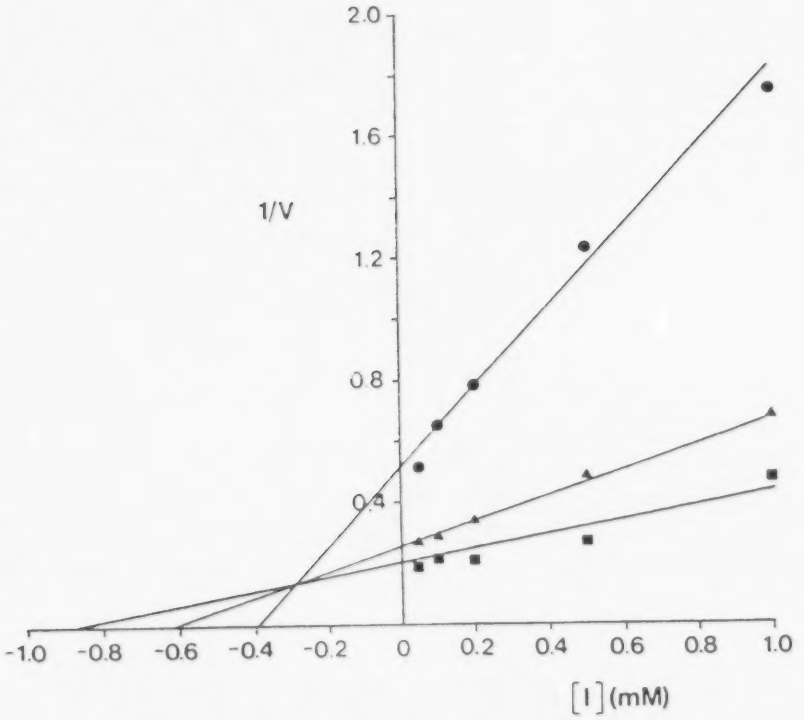
Sequential inhibitor analysis



The effect of BCH, tryptophan, and the sulfur homolog of tyrosine, 4-S-cysteinylphenol (4-S-CP) on tyrosine transport in SK-MEL 23 cells was studied. The SK-MEL 23 cells were incubated with 0.1 mM tyrosine and a) increasing concentrations of BCH, 2.25 mM BCH plus increasing concentrations of 4-S-CP, or 2.25 mM BCH plus 10 mM 4-S-CP plus increasing concentrations of tryptophan added simultaneously (■ ;BCH/4-S-CP/tryptophan). b) Studies the effects of the same compounds on tyrosine transport, but reverses the order of BCH and tryptophan addition (● ; tryptophan/4-S-CP/BCH). Under all conditions the cells were incubated for 20 seconds then washed 3 times with ice-cold buffer. The cells were then lysed using 0.2 N NaOH and samples taken for determination of [^{3}H]-tyrosine by liquid scintillation and protein content by the Bradford assay. Control value was 5.38 ± 0.37 nmol/min/mg protein. Values are means ± SE of triplicate determinations. The lack of error bars represents errors so small that they fall within the points.

Figure 3.7

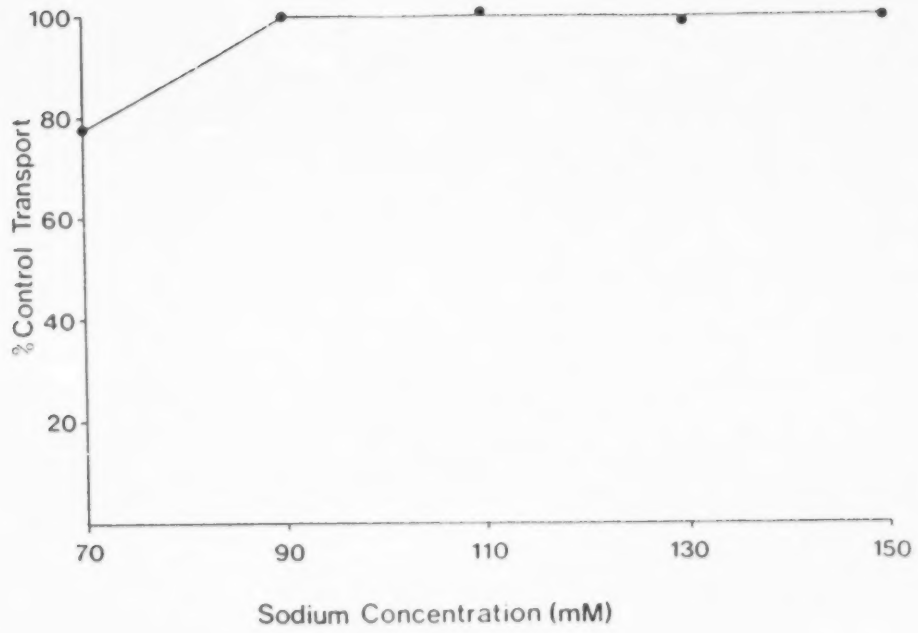
Dixon plot to determine the effects of 4-S-CP on tyrosine transport



A Dixon plot using tyrosine concentrations of 0.05 mM (●), 0.1 mM (▲), and 0.2 mM (■) with 4-S-CP concentrations of 0.05-1.0 mM were used to determine the effect of the addition of this compound on tyrosine transport. Cells were incubated for 20 seconds in the supplemented transport buffer. The cells were then washed 3 times with ice-cold buffer then lysed using 0.2 N NaOH and samples taken for determination of [^{3}H]-tyrosine by liquid scintillation and protein content by the Bradford assay. Values are means ± SE of triplicate determinations. The lack of error bars represents errors so small that they fall within the points. The K_i of this competitive inhibition was determined to be 0.3 mM based on this data.

Figure 3.8

Effects of changing osmolarity of the uptake medium on tyrosine transport



Decreasing concentrations of NaCl without compensation for the loss of those ions produces a hypoosmotic uptake medium. The effects on tyrosine transport were determined at 70-150 mM NaCl concentrations in the presence of 0.1 mM tyrosine. Cells were incubated for 20 seconds in the tyrosine supplemented transport buffer. The cells were then washed 3 times with ice-cold buffer then lysed using 0.2 N NaOH and samples taken for determination of [^{3}H]-tyrosine by liquid scintillation and protein content by the Bradford assay. Control value was 4.29 ± 0.40 nmol/min/mg protein. Values are means ± SE of triplicate determinations. The lack of error bars represents errors so small that they fall within the points.

CHAPTER 4
DISCUSSION

4.1 CHARACTERIZATION OF TYROSINE TRANSPORT

Tyrosine is an amino acid that has a unique function in melanocytes and melanoma cells. In addition to its involvement in general protein synthesis, tyrosine is required for the production of the melanin pigments, eumelanin and pheomelanin. Therefore, a better understanding of the mechanism of tyrosine transport in pigment producing cells will have implications not only for the understanding of melanogenesis, but also for the development of new chemotherapeutic agents based on the melanin synthetic pathway.

The kinetic data from this study, obtained using a monolayer-cell wash assay procedure, indicate that the tyrosine transport in SK-MEL 23 human melanoma cells has a K_m of 0.164 ± 0.016 mM and a V_{max} of 21.6 ± 1.1 nmol/min/mg protein (mean SE). In the three studies where the effects of NaCl and KCl medium can be compared, ie., time course, inhibition, and NaCl replacement, there appears to be little change in the transport characteristics in the absence of Na^+ (Figure 3.1 and Table 3.1,3.2). Therefore, tyrosine transport in this cell line can be

classified into the Na^+-independent category of amino acid transport systems.

Specific inhibitors can be very useful in determining the nature of amino acid transport (43,44,81). If it can be demonstrated that there is a decrease in transport because of the presence of another compound, this compound can be assumed to act on a similar transport system. Thirteen different potential inhibitors were tested for their effects on tyrosine transport. Inhibition with the system A specific substrate MeAIB and the system ASC substrate AIB was minimal (Table 3.1). Both of these transport systems are also Na^+-dependent, negating any direct involvement with tyrosine transport in this cell line because of the Na^+-independent nature of the transport.

Phenylalanine had previously been shown to decrease tyrosine incorporation into melanin by preventing the uptake of tyrosine in RPMI-3460 hamster melanoma cells (82). In this study phenylalanine, which can be transported by either system L or system T, produced 77.1% inhibition of tyrosine transport in the human melanotic melanoma cell line SK-MEL 23.

BCH inhibition is a characteristic feature of system L transport because of its selectivity for only this transporter (44). In this study, the addition of BCH to

the uptake medium caused 63.0% inhibition of tyrosine transport in the SK-MEL 23 melanoma cells.

Tryptophan is the characteristic substrate for system T in erythrocytes (61,62) and hepatocytes (11), but can also be transported by system L in other cell types (47,48). In the SK-MEL 23 melanoma cell line, tryptophan inhibition of tyrosine transport has characteristics similar to those of BCH in every comparable situation (Figure 3.5,3.6 and Table 3.1). Both BCH and tryptophan had similar concentration effects in the single inhibition experiment (Figure 3.5) and in the sequential inhibition experiment (Figure 3.6). System L and system T are both capable of transporting aromatic amino acids and are known to have overlapping specificities (44,61). It appears that the SK-MEL 23 cells may utilize a single transport system for the aromatic amino acids.

The residual flux that remains after maximal inhibition has been reached (Figure 3.6) is probably due to diffusion or involvement of a H^+ dependent transporter (83,84). This residual flux represents less that 10% of the tyrosine transport observed in this cell line.

All of the present findings, ie., substrate specificity, Na^+-independence, inhibitor analysis, and kinetics, indicate that greater than 90% of tyrosine transport into the human melanotic melanoma cell line,

SK-MEL 23, is similar to that previously described as system L. This transport also appears to be relatively unaffected by rather large changes in the osmolarity of the growth medium. Tyrosine transport in B16 murine melanoma cells has recently been studied and found to be similarly transported by a single mechanism, system L (49). A second set of studies by Jara et al (85,86) concludes that both system L and ASC may be involved in tyrosine transport under different conditions in B16/F10 murine melanoma cells. These studies used methodologies and cells lines that differed from those presented here which prevents a direct comparison of all results. However, it is apparent that tyrosine transport into these melanin producing cells is an essential step in the melanogenesis pathway.

4.2 TRANSPORT AND TOXICITY

Malignant melanoma remains one of the most challenging cancers to treat successfully (22). It has been found to be very resistant to radiation and current chemotherapy regimens making most of the conventional treatments ineffective. Several different approaches have been attempted in an effort to develop a new class of chemotherapeutic agents against melanoma. One approach, which has been used with some success in other treatments, is the use of analogs of natural compounds.

Analogs of amino acids have been used as chemotherapeutic agents with the transport of these analogs into various cell types being implicated, at least partially, in their efficacy. One example is the L-phenylalanine mustard, melphalan, used clinically in the treatment of multiple myeloma (66) and malignant melanoma (67). Melphalan transport has been characterized in normal human lymphoblasts and bone marrow cells (68), cells of human breast and ovarian cancer (68), as well as in murine leukemia cells (66,69). Its effectiveness as a cytotoxic agent in these cell lines has been associated with its transport through amino acid transporters, particularly system L and system ASC.

In a similar fashion, the sulfur homolog of tyrosine, 4-S-CP, has been synthesized in an effort to utilize the melanogenic pathway in the treatment of melanoma (27). By using the tyrosine like compound, it may be possible to produce cytotoxic intermediates intracellularly capable of melanocytic destruction. 4-S-CP, along with other tyrosine homologs, was previously shown to be a substrate of the specific melanin producing enzyme tyrosinase and capable of increasing the life span of melanoma-bearing mice and causing growth inhibition of melanoma tissue <u>in vivo</u> (26,27,87,88).

Using [^{3}H]-4-S-CP, Nakamura et al (70) were able to show that this compound can be selectively incorporated into a human melanotic melanoma cell line, HMV II, *in vitro*. Through a whole body autoradiography study, [^{3}H]-4-S-CP was also shown to accumulate in subcutaneous B-16 melanoma tumors grown in C57BL/6J mice (26,28). In this present study, 4-S-CP was a very good inhibitor of tyrosine transport, resulting in 61.2% inhibition at a 1.0 mM concentration. The inhibition was comparable to the inhibition observed with the other aromatic and neutral amino acids in particular the system L specific leucine analog, BCH.

The K_i for the competitive inhibition of 4-S-CP on tyrosine transport was determined to be 0.3 mM based on the Dixon plot (Figure 3.7). These results would indicate that 4-S-CP crosses the membrane in a fashion similar to that of tyrosine, and that manipulation of tyrosine transport may alter the effects of this potential chemotherapeutic agent.

4.3 REGULATION OF TRANSPORT

Animal cells adapt to fluctuations in extracellular amino acid availability or intracellular requirements by regulating the activation of certain transport systems (62). As cells mature, their requirements for various essential nutrients change with the mechanism of

transport of these compounds also being affected. Using a temperature sensitive Chinese hamster ovary mutant cell line, Shotwell et al was able to show adaptive changes in system L transport (44,89,90). When these cells were grown at less than optimal conditions, the transport activity for leucine increased 2-3 fold. This transport enhancement was reflected by an increase in the V_{max} of leucine uptake with no change in its K_m suggesting an increase in the number of system L transport carriers. The transport enhancement could be prevented by the addition of the protein synthesis inhibitor, cycloheximide, but not by actinomycin D, an inhibitor of transcription (90).

Lobaton et al (91), using a Chinese hamster-human hybrid temperature sensitive cell line was able to demonstrate similar changes in system L transport. Chromosome analysis led to the conclusion that the high leucine transport phenotype was correlated with the loss of human chromosome 20 in this hybrid cell line.

Control by hormones is one mechanism by which nutrient flows are coordinated in multicellular organisms. Amino acids are nutrients whose levels are affected by the addition of hormones and other compounds that alter cellular metabolism (92,93). In most cases, there is a correlation between the stimulation of amino acid uptake by a hormone in its specific target tissue

and an increase in the metabolic activities of the cell such as macromolecular synthesis.

There exists a wide variety of hormones that are capable of stimulating amino acid transport suggesting that there are several different routes by which the hormone-mediated stimulation of transport can occur (89,92). The addition of hormones may result in de novo synthesis of transport proteins. The use of the protein synthesis inhibitors puromycin or cycloheximide has shown that the action of some hormones is on the synthesis of transport proteins. The role of protein synthesis may be to produce new transport molecules or alternatively to alter the levels of factors that regulate transport activity.

The activation of inactive carriers or the recruitment of membrane transport carriers are other possible mechanisms for the hormone-mediated stimulation of amino acid transport. A second protein synthesis-independent mode of transport stimulation might occur by post-translational modification of a protein component of the transport protein (eg. protein phosphorylation).

For example, the addition of hormones insulin and glucagon can affect amino acid transport in hepatic cells in vitro and in vivo (92). Glucagon, possibly via cAMP, increased the hepatic uptake of the system A substrates AIB and MeAIB. Insulin, acting through a separate

mechanism, stimulated a transport system that had no affinity for MeAIB but increased AIB incorporation. Both of these naturally occurring hormones affected the transport of amino acids by altering the intracellular second messenger systems and both stimulated changes in membrane protein phosphorylation (92,94).

Leucine transport in human B lymphocytes is primarily via system L (56). TPA treatment of B lymphocytes from patients with chronic lymphocytic leukemia resulted in an increase in BCH-inhibitable leucine uptake (system L). The intracellular effects of TPA have been found to be mediated by the stimulation of the intracellular effector molecule, protein kinase C.

The potential toxicity of tyrosine for melanocytes make it likely that the availability of tyrosine for melanin synthesis is under stringent control. Due to the unique role of tyrosine in melanocytes and melanoma cells, it may be possible to alter its transport by altering the specific metabolic pathway of melanogenesis. Various compounds have been used to either decrease or enhance melanin synthesis <u>in vitro</u> and <u>in vivo</u>.

PTU has been used as a specific inhibitor of the enzyme tyrosinase (80). PTU inhibits tyrosinase function by entering the cells and combining directly with the enzyme resulting in the loss of tyrosinase function and a lack of pigment production in the cells. However, in

this study PTU addition did not affect tyrosine transport when compared to the untreated control (Table 3.3). Therefore, the inhibition of tyrosinase alone may not be enough to alter the tyrosine transport.

MSH has been used in a number of different melanocyte and melanoma cell lines to increase melanin production (7,11). It acts by stimulating adenylate cyclase which results in increased intracellular cAMP levels. The affects of the increased cAMP levels in pigment producing cells is not yet fully understood, but it is believed that it acts through cAMP dependent protein kinases to alter melanogenesis (7,11). Wong and Pawelek (95) were able to show that treatment of a murine melanoma cell line, Cloudman S91, with MSH resulted in an activation of pre-existing tyrosinase molecules via cAMP. When the SK-MEL 23 cells were grown in the presence of a stimulating concentration of MSH and then tested in the tyrosine transport assay, there was an increase in the tyrosine transport as compared to an untreated control cell culture (Table 3.3).

It is possible that cAMP or cAMP-dependent protein kinases are involved in the increase through the stimulation of synthesis of transporters, by increasing the number of transporters already present at the membrane, by increasing the turnover rate of these transporters already present at the membrane, or by the

recruitment of transporters not originally responsible for tyrosine transport. Any of these situations would result in an increase in the tyrosine transport in melanoma cells after stimulation with MSH.

PTU acts upon a single enzyme to attain its final effects. MSH stimulation results in the activation of the cAMP cascade which potentially could have multiple intracellular functions. The results indicate that inhibiting tyrosinase alone is not enough of a stimulus to alter cellular transport of tyrosine. It may be that more than a simple change in enzyme function is required for an alteration in transport.

4.4 POSSIBLE HORMONAL FUNCTION OF TYROSINE

Tyrosine and dopa themselves have also been suggested to act as regulators of the melanogenic apparatus in a similar fashion to that of MSH (13,18,19,96). This would mean that tyrosine and dopa may have hormone-like function in the melanocytes and melanoma cells in addition to their traditional role as an enzyme substrate. Evidence is accumulating that, in vertebrates, tyrosine and dopa can act as inducers of the melanogenic apparatus, expression and activity of MSH receptors, melanocyte proliferation, and of energy yielding metabolism (18-20). In cultured amelanotic hamster melanoma cells, the addition of tyrosine to the

growth medium induces melanogenesis and synthesis of melanogenesis proteins such as tyrosinase, structural proteins and auxiliary enzymes.

To act as a hormone, membrane receptors for these compounds would be required for the activation of an intracellular response. Tyrosine receptors have not been detected in multicellular organisms, but have been documented in unicellular organisms (97). One potential candidate as a receptor for tyrosine and dopa is the transmembrane protein tyrosinase (13). It has separate tyrosine and dopa binding sites, and melanosome specific proteins can be expressed on the cell surface. However, since tyrosinase is usually localized to the melanosome it is unlikely that there would be enough of the enzyme present on the cell surface to account for the tyrosine transport into the cell. Still tyrosinase, or other melanosomal membrane proteins, may be important for the localization of tyrosine to the site of melanin synthesis, the melanosome.

4.5 MELANOSOME AND TRANSPORT

Any transport system serving largely for amino acids of special metabolic significance in a given tissue or cell type may be expected to show some degree of functional specificity to that tissue or cell type (45). In melanocytes the special metabolic requirement is the

synthesis of melanin pigments that takes place inside the melanosome. In order for melanogenesis to occur, tyrosinase inside the melanosome must be active and the appropriate melanin precursors must be present. Consequently, it may be the melanosomal membrane that assumes ultimate control over the type of melanin synthesized by the cell. If the melanosome will not permit cysteine or sulfhydryl compounds to come into contact with the melanin intermediates, then pheomelanin cannot be synthesized. If tyrosine is incapable of entering the melanosome, then the whole process of melanogenesis may not be initiated due to a lack of substrate.

The melanosomal membrane may be responsible for the selectivity of the substrates and therefore for the type and amount of melanin produced by the cell. There are at least 15-16 polypeptides that have been identified as components of the melanosome (2). Only a few of these have been characterized as to their function, leaving many as yet undetermined with relation to their role, if any, in melanogenesis. One or more of these proteins may be associated with the regulation of melanosomal membrane transport capable of determining the nature of the melanin pigment produced by the cell and possibly involved in the control of cell growth and differentiation as a result.

System L transport has been shown to be a weakly concentrating system, unable to develop large concentration gradients across the membrane due to the wide range of compounds it transports and the exchange capabilities of this transport system (43-46). Consequently, tyrosine can gain access to the cytoplasm of melanin producing cells, but would serve as a general pool for all cellular functions. The presence of melanosomal amino acid transporters could more significantly contribute to the localization and regulation of melanin synthesis by controlling the flow of precursors into the melanosome.

The presence of these melanosomal transporters could also explain the selective accumulation of the tyrosine homolog, 4-S-CP, in the melanin synthesizing tissues. Once the compound has gained access to the inside of the cell via system L transport, melanosomal membrane proteins may allow for the transport of the compound into melanosomes. Here the metabolic machinery can metabolize the compound and therefore allow for accumulation at this location. This compartmentalization would allow for an increase in substrate concentration at the melanin synthesis site, while still allowing an adequate supply of amino acids for general cellular protein synthesis.

Future studies will need to determine the nature of the melanin precursor transport into the melanosome, and

its role, if any, in the regulation of melanin synthesis and the development of specific chemotherapeutic agents.

CHAPTER 5
CONCLUSIONS

The transport of tyrosine in the human melanoma cell line SK-MEL 23 was found to be mediated by a single, Na^+-independent transport system, system L. The K_m and V_{max} values of this transport system were 0.164 0.016 mM and 21.6 1.1 nmol/min/mg protein (mean SE) respectively. Modification of the melanin synthetic pathway, a biochemical pathway dependent on tyrosine and unique to pigment producing cells, may allow for the study of tyrosine transport regulation. Inhibition of the melanin synthesizing enzyme, tyrosinase, was not a sufficient stimulus to decrease the tyrosine transport. Treatment of the SK-MEL 23 cells with the melanogenic hormone, MSH, resulted in increased tyrosine transport. MSH has been shown to act via cAMP, which may have numerous intracellular effects that could result in increased tyrosine transport. The nature of the changes necessary for tyrosine transport regulation as well as the cause for the accumulation of tyrosine homologs in melanin producing cells are important areas for future research in the development of new anti-melanoma agents based on this unique biochemical pathway.

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UNIVERSITY OF ALBERTA

TYROSINE TRANSPORT IN A HUMAN MELANOMA CELL LINE

BY

JAMES MILAN PANKOVICH

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

IN

Experimental Medicine

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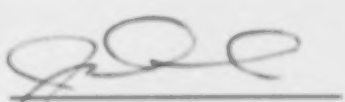

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Tyrosine Transport in a Human Melanoma Cell Line submitted by James Milan Pankovich in partial fulfillment of the requirements for the degree of Master of Science in Experimental Medicine.

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August 11, 1992

ABSTRACT

Tyrosine is an amino acid essential for the initial step of melanin synthesis, yet little is known concerning its transport in melanocytes. As an important first step in the development of new anti-melanoma agents based upon exploitation of the melanin synthetic pathway, this study characterized the transport mechanism of tyrosine in vitro using the human melanoma cell line, SK-MEL 23. Several tyrosine transport systems may be involved in melanocytes, systems L, T, $B^{o,+}$, and $b^{o,+}$ which transport neutral amino acids with branched or aromatic side chains, and systems A, ASC, and asc which transport neutral amino acids with smaller side chains. In order to determine which system or combination of these is involved in tyrosine transport by melanoma cells, kinetics, Na^+ dependence, and competitive inhibition studies were undertaken. The K_m and V_{max} for the Na^+-independent transport system were found to be 0.164 ± 0.016 mM and 21.6 ± 1.1 nmol/min/mg protein (mean ± SE) respectively. This transport was preferentially inhibited by the system L specific analog, 2-aminobicyclo-(2.2.1)-heptane-2-carboxylic acid (BCH), the system T substrate tryptophan, and the sulfur homolog of tyrosine, 4-S-cysteinylphenol (4-S-CP). Sequential addition of these inhibitors at increasing concentrations indicated that they may inhibit the same transporter.

Our results suggest that tyrosine transport in the SK-MEL 23 melanoma cells is similar to system L transport previously characterized in other cell types. This transport system appears to supply all the tyrosine required for both cell growth and melanin synthesis. The transport system may be subject to manipulation by melanogenic stimulating factors making the transport of cytotoxic tyrosine analogs an important area for further study.

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List of Abbreviations

UV	- ultraviolet light
5,6-DHI	- 5,6-dihydroxyindole
5,6-DHICA	- 5,6-dihydroxyindole-2-carboxylic acid
5-S-CD	- 5-S-cysteinyldopa
dopa	- 3,4-dihydroxyphenylalanine
MSH	- melanocyte stimulating hormone
cAMP	- cyclic adenosine monophosphate
4-S-CP	- 4-S-cysteinylphenol
4-S-CAP	- 4-S-cysteaminylphenol
PTU	- phenylthiourea
BCH	- 2-aminobicyclo-(2.2.1)-heptane-2-carboxylic acid
AIB	- 2-aminoisobutyric acid
MeAIB	- 2-methylaminoisobutyric acid
MEM	- minimal essential medium
FBS	- fetal bovine serum
EDTA	- ethylenediaminetetraacetic acid
dpm	- disintegrations per minute
BSA	- bovine serum albumin
HEPES	- N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
TPA	- 12-O-tetradecanoylphorbol 13-acetate

CHAPTER 1
INTRODUCTION

1.1 MELANOCYTES AND PIGMENTATION

Melanocytes are cells capable of synthesizing the enzyme tyrosinase which, when incorporated within specialized intracellular organelles, the melanosomes, initiates events leading to the synthesis and deposition of melanin (1). This definition of the metabolic machinery leading to melanin synthesis highlights the various unique features of the melanocyte. Those unique features may provide the basis for biological and physiological studies of properties related to the transport and regulation of substrates necessary for the synthesis of the pigments. The same features may provide the basis for establishment of specific chemotherapy of the neoplastic form of melanocytes, melanoma cells, in as much as this enzyme-substrate interaction could generate cytotoxic intermediates.

Embryologically melanocytes arise from the neural crest (Figure 1.1). In humans, melanocytes are present in the skin at the dermo-epidermal junction, in hair follicles and to a lesser extent in the lower layer of the skin, the dermis (2). In addition, melanocytes are

also found in the nervous system, eye, and inner ear where they are located in the dermis or among the connective tissues.

Melanocytes of the dermo-epidermal junction and hair follicles are responsible for the production of melanin pigments that will be a major factor in determining the color of skin and hair. Melanocytes discharge their melanin pigment granules into the surrounding keratinocytes to give the skin or hair visible color. Most of the extracutaneous melanocytes are comparable to dermal melanocytes in that they do not discharge melanin pigments into surrounding cells, but rather retain the pigments.

Normal human skin color - red, yellow, brown and blue - is produced by four skin pigments: by exogenously produced carotenoids (yellow) and endogenously produced melanin (brown) in the epidermis, by oxygenated hemoglobin (red) in the capillaries, and by reduced hemoglobin (blue) in the venules of the dermis (3). Of these, melanin is the major determinant of differences in skin color between individuals (3). It is the amount of melanin that has been transferred to the keratinocytes from the melanocytes that significantly determines the pigmentation of the skin and hair.

Melanin pigmentation of human skin can be divided into two components (2). Constitutive skin color is the

amount of cutaneous melanin pigmentation generated according to cellular genetic programs without any direct influence of external factors such as solar radiation. Facultative skin color or "tan" is elicited by direct exposure of the skin to ultraviolet (UV) light, or other regulators of melanin synthesis. Facultative skin color is reversible in that the skin responding to the stimulus will return to the original color upon removal of the stimulus. These color changes characterize the immediate and delayed tanning reactions induced by exposure to UV light.

1.2 MELANINS

There are two major classes of integumentary melanin formed by the action of tyrosinase: the black-brown nitrogenous eumelanin and the yellow to red, sulfur containing pheomelanin (4,5). Despite the obvious color differences these pigments are biologically related, and both arise from a common metabolic pathway in which tyrosine is the initial substrate and dopaquinone is the key intermediate (Figure 1.2).

Eumelanin is best characterized as a highly irregular, three-dimensional polymer of several types of monomers joined covalently into an integrated structure (1,5). The most common monomers are 5,6-dihydroxyindole (5,6-DHI) and 5,6-dihydroxyindole-2-carboxylic acid (5,6-

DHICA). Eumelanin has the characteristic of being insoluble in almost all solvents.

Pheomelanin appears in nature by a modification of the eumelanin pathway which involves the interaction of the amino acid, cysteine, or the tripeptide, glutathione, with dopaquinone, an intermediate produced by enzymatic oxidation of tyrosine. Chemically, pheomelanin is distinguishable by its relatively high content of sulfur which results from the addition of cysteine to dopaquinone to yield a 5-S-cysteinyldopa (5-S-CD) compound (1,4,5). This 5-S-CD monomer can then polymerize to form the yellow-red pigment.

Pure pheomelanin is formed only by cysteinyldopa oxidation products and pure eumelanin is formed only by indoles. In nature, these pure melanin products may be quite rare. Instead, most pigments seem to be mixed-type melanins which are formed by copolymerization of oxidation products of 3,4-dihydroxyphenylalanine (dopa) alone and dopa plus cysteine (1). The quantities of the intermediates available at the site of polymerization will determine the type of melanin formed. As well, melanin is not simply a polymer of oxidized tyrosine, but may contain proteins of undefined structure and function. These include structural proteins of the pigment granules and other proteins that are present in the melanosomes (5).

There is a wide range of physiological roles suggested for the melanins including protection of underlying tissues from UV radiation, heat control, and adaptive coloration (4,6). These may be evolutionarily conserved functions of a process that originally might have had a more fundamental biological significance than the provision of a visible pigment.

As early as 1820, Sir Everard Home first proposed a concept that melanin acts as a sunscreen (6). Today most pigment cell biologists believe that the primary function of melanin pigments in humans is as a sunscreen to protect the skin from the UV radiation in sunlight even though melanin may not fulfill all the properties of an ideal sunscreen.

1.3 MELANOSOMES

Within melanocytes, melanin is formed by the tyrosine-tyrosinase reactions and subsequent cyclizations of the products within intracellular organelles, the melanosomes. The formation of melanosomes involves the assembly and organization of four basic components that include membranous phospholipids, structural proteins, the essential enzyme tyrosinase, and possibly auxiliary enzymes (Figure 1.1).

Four stages of melanosome development are recognized in both eumelanogenesis and pheomelanogenesis (2). Stage

I melanosomes in eumelanogenesis are spherical vacuoles containing amorphous, proteinaceous materials and a few microvesicles also termed vesiculoglobular bodies. Stage II eumelanosomes take on a characteristic ellipsoidal shape and are assembled with organized lamellae of filaments and vesiculoglobular bodies. Tyrosinase activity is not detectable with the granules of stage I and II melanosomes by conventional techniques. In stage III eumelanosomes, tyrosinase activity becomes detectable and melanization begins on the lamellae of the inner matrix. Stage IV eumelanosomes are highly melanized and electron dense under electron microscopic examination.

Pheomelanosomes are always spherical and do not form a lamellar or fibrillar matrix such as seen in eumelanosomes. Instead, they conjugate together with numerous vesiculoglobular bodies or microvesicles in all stages of melanosomal development. The difference in morphology between pheomelanosomes and eumelanosomes may simply be based on a different organization of the same ingredients under the influence of sulfhydryl compounds (2).

1.4 TYROSINASE

Tyrosinase is a copper containing enzyme responsible for the production of melanin pigments from the initial substrate tyrosine. There has been a continuing

controversy whether there exists one enzyme with two functions or a two enzyme system, ie., tyrosinase plus another oxidation enzyme, for melanin synthesis. Evidence now seems to indicate that only one enzyme with two functions needs to be present to produce melanin (3,7). This single enzyme catalyzes the hydroxylation of tyrosine to dopa and subsequently the oxidation of dopa to dopaquinone. Melanin can be formed from dopaquinone without further enzyme participation. More recently, an enzyme, dopachrome conversion factor, has been identified that may act downstream from tyrosinase to increase the efficiency of the polymerization process (8).

Tyrosinase is initially transcribed on membrane bound ribosomes, transported through the endoplasmic reticulum to the Golgi region, where it is modified, packaged into vesicles, and then transported to melanosomes (Figure 1.1) (1). These tyrosinase containing vesicles then fuse with the melanosomal membrane to produce a stage III melanosome. The tyrosinase becomes activated in an as yet unknown manner, and melanin formation and deposition can proceed.

Tyrosinase can be present in multiple forms, T_1, T_2, T_3, all possessing tyrosine hydroxylase and dopa oxidase activity (4,9,10). These different forms probably represent the same core molecule at different stages of post-translational glycosylation (1,9). The core protein

has an apparent molecular weight of 55 kDa, and remains kinetically unchanged during its subsequent processing. Post-translational modifications may be necessary to assure the delivery and orientation of tyrosinase in the melanosome (9).

1.5 MELANOGENESIS

Having introduced the major components involved in melanin synthesis, it is important to understand how they all are integrated in a biological system to produce melanin pigments.

Even though many of the details of melanin synthesis are still to be worked out, it seems that the rate-limiting step in melanogenesis occur at the level of the oxidation of tyrosine to dopa. This conversion of tyrosine to dopa occurs inside the melanosomes and is mediated by the enzyme tyrosinase. Both types of melanin, eumelanin and pheomelanin, share this common step in the metabolic pathway (Figure 1.2). Eumelanin will be produced if dopaquinone proceeds to cyclize and oxidize. If, during the conversion of tyrosine to melanin, dopaquinone encounters cysteine or other sources of sulfhydryl groups, pheomelanin will be the major product.

The process of melanin synthesis is under the control of multiple genes, with more than 150 loci having

been implicated in mice. Thus, there are potentially many factors that can affect the rate and type of melanin synthesis.

Traditionally, melanocyte stimulating hormone (MSH) has been used to increase melanization of pigment producing cells in vitro (11,12). The increased melanization in response to MSH is mediated through the intracellular second messenger cyclic adenosine monophosphate (cAMP) (7,11). MSH is able to bind to receptors present on the cell surface and the formation of this receptor complex stimulates the activity of adenylate cyclase, which in turn leads to increases in the intracellular levels of cAMP.

MSH receptors are the best characterized regulatory elements governing pigmentation, morphology, and proliferation of pigment cells in vertebrates (13). The cells regulate their response to MSH by the transient appearance of cell membrane receptors for this hormone signal (7). An increase in tyrosinase activity can be detected 8 hours after MSH stimulation, and melanin formation takes place about 16 hours after the initial MSH challenge (12). MSH can also modify cellular proliferation and alter the morphology of melanoma cells in culture.

Exposure of melanocytes to an external regulator, UV radiation, leads to changes in melanin synthesis, the so

called tanning reactions (14-16). UV radiation results in an increase in melanocyte number by an undefined molecular mechanism and, subsequently the overall rate of synthesis of the enzyme (the long term effect). It also has a short term effect, ie., the rapid polymerization of pre-existing melanin precursors within the melanosome which occurs within a matter of minutes. Both of these effects result in overall increased pigment production. The UV radiation-mediated activation of melanogenesis has been found to be due to the activation of cAMP-independent mechanisms, perhaps involving the inositol triphosphate or diacylglycerol second messenger pathways (14,15,17).

There is recent evidence that, at least in vertebrates, tyrosine and dopa themselves can act as inducers and regulators of the melanogenic apparatus, the expression and activity of MSH receptors, and melanocyte proliferation (13,18-20). Supplementation of the growth medium with tyrosine or dopa during the culturing of amelanotic melanoma cells results in a rapid increase in melanin formation, which is not simply due to greater availability of substrate (18,19). Positive regulation of melanogenesis-related proteins following addition of tyrosine has been shown (20). Upon treatment with tyrosine in the culture medium there is a rapid increase in tyrosinase activity and large scale synthesis of

melanosomes. These two events would combine to increase the synthesis of melanin.

1.6 RATIONAL CHEMOTHERAPY

Malignant melanoma is a cancer with increasing worldwide incidence (21). At the present time, the primary treatment for localized melanoma is surgical excision. Prognosis worsens as the disease spreads with the median survival after diagnosis of metastasis being less than 5 months (22). Radiation and existing methods of chemotherapy are limited in their efficacy. Consequently there is an urgent need for new and improved treatments for malignant melanoma.

Since 1963 when Hochstein and Cohen (23) proposed that polyphenolic intermediates in the formation of melanin from tyrosine are potentially cytotoxic agents, many attempts have been made to try and exploit this biochemical pathway for the treatment of melanoma. Most attempts to develop chemotherapeutic agents specific to malignant melanoma have involved the exploitation of intermediates of melanin synthesis, agents which are assumed to enhance melanin pigmentation, or a combination of these two.

Ideally a chemotherapeutic agent should selectively discriminate between the normal and target neoplastic cell type to minimize the destruction of normal cells

essential for the survival of the host. This principle may not necessarily apply to melanoma chemotherapy. Melanocytes serve an apparently non-essential role in the host, therefore the destruction of normal melanocytes along with melanoma cells would be insignificant when compared to the manifestations of the disease itself. The specificity is important is being able to distinguish between melanin and non-melanin producing cells.

Melanocytes and melanoma cells both contain the unique enzyme tyrosinase. This enzyme is capable of catalyzing the hydroxylation of tyrosine to dopa and the oxidation of dopa to dopaquinone. These are the first two steps in the melanin synthetic pathway. The enzyme tyrosinase provides these melanin producing cells with a specific biochemical target to exploit in the development of chemotherapeutic agents. Since this enzymatic activity is restricted to melanocytes and generally present in larger amounts in melanoma cells (24), a defined biochemical target is present. The rationale of this approach is not necessarily to interfere with melanin biosynthesis since melanin production is not essential to the general metabolic economy of the melanoma cell, but rather to design compounds which are converted to cytotoxic derivatives by the action of a unique enzymatic apparatus within the melanoma cell. It is known that even some of the natural melanin precursors

and intermediates are cytotoxic and that cells actively producing melanin run the risk of self-destruction through a build up of these precursors (25).

Numerous melanin precursors have been explored as possible chemotherapy agents. These may be grouped into one of three categories and considered to be related to either tyrosine (phenolic), 3,4-dihydroxyphenylalanine (catecholic), or 5,6-dihydroxyindole (intermediates).

Tyrosine serves as the starting material for the biosynthesis of melanin and is also an important substrate for melanoma cells in vitro. Therefore, in vitro the degree of pigmentation can be influenced by changing the concentration of tyrosine in the growth medium. Analogs of the amino acid tyrosine have been developed as potential chemotherapeutic agents. This group of phenolic melanin precursors include the sulfur homolog of tyrosine, 4-S-cysteinylphenol (4-S-CP), its amine derivative, 4-S-cysteaminylphenol (4-S-CAP) (26-28), and 4-S-CAP derivatives (28,29). Both 4-S-CP and 4-S-CAP have been tested and shown to increase the life span of melanoma bearing mice and to cause the in vitro growth inhibition of melanoma tissue (27-30). Subcutaneous injections of 4-S-CP and 4-S-CAP into C57BL/6J black mice caused the depigmentation of the black hair (31), and topical application of these compounds to black guinea pigs resulted in depigmentation

of the skin (32). In both cases, the depigmentation was manifested by the loss of functioning melanocytes.

3,4-Dihydroxyphenylalanine (dopa) and its structural analogs are also known to be cytotoxic to melanoma cells. Dopa, dopamine, dopa methyl ester, norepinephrine, 6-hydroxydopamine, and 3,4-dihydroxybenzylamine were studied for their effects on human and murine melanoma cells in vitro (33,34). Both dopa and dopamine analogs are capable of inhibiting the growth of a variety of cell lines.

Two mechanisms have been proposed concerning the cytotoxicity of catechols. The first is an intracellular mechanism in which catechols are converted to the corresponding benzoquinones by tyrosinase, and these quinones are then believed to cause damage to cells through inactivation of sulfhydryl enzymes essential for cellular function (34,35). An alternative mechanism involving catechols has them being auto-oxidized extracellularly by the interaction with amine oxidases to produce cytotoxic hydrogen peroxide, superoxide, and hydroxyl radicals (33,34,36-38). This second mechanism can result in systemic rather than specific toxicity, decreasing the efficacy of the compounds as chemotherapeutic agents. The major limitations of dopa derivatives appear to be: 1) instability, 2) insufficient lethality of dopa toward neoplastic cells in

vivo, and 3) low physiologically tolerable dosages of 6-hydroxydopa and other catechols due to general cytotoxicity (26).

Intermediates in the melanin synthetic pathway are also potential chemotherapeutic agents. 5,6-Dihydroxyindole, an intermediate in the eumelanin pathway, has been shown to be toxic to melanoma cells in vitro (39). The catecholic amino acid, 5-S-CD, a major intermediate of pheomelanin, is also toxic to a variety of human tumor cell lines including murine L1210 leukemia and B16 melanoma (40,41). These compounds are difficult to synthesize and are quite unstable making them unlikely candidates for therapeutic agents.

Phenolic melanin precursors, that may structurally resemble the natural substrate tyrosine, provide a rational approach to the design of antitumor agents for melanoma. Many previous attempts at developing chemotherapeutic agents against melanoma have been based upon dopa and related catecholic compounds that may cause general cytotoxicity from auto-oxidation (26,33,34). Alternatively phenolic compounds, such as 4-S-CP and 4-S-CAP, have been found to be more potent melanocytotoxic agents than catechols using an in vivo assay system (31). Another phenolic compound, 4-hydroxyanisole (4-OHA), has also been shown to have cytotoxic effects on melanocytes

and melanoma cells, but its toxicity is probably due to oxygen radicals formed extracellularly (42).

If analogs of tyrosine could be taken up by melanoma cells and oxidized intracellularly to cytotoxic quinones, then these neoplastic cells could be selectively killed while sparing normal tissues that do not have tyrosinase. The conversion of these phenolic compounds to toxic intermediates should also be enhanced by increasing the tyrosinase activity of the cell. Tyrosinase can be activated in response to elevated levels of cAMP in a reaction apparently mediated by cAMP-dependent protein kinases (39). Increases in intracellular cAMP levels can be achieved through the administration of the hormone MSH. Cells that had an artificially high tyrosinase activity that resulted from exposure to MSH were much more susceptible to the toxicity of melanin precursors (39). On the other hand, inhibition of tyrosinase should result in a decrease in toxicity. Treatment of cells with phenylthiourea (PTU), a potent inhibitor of tyrosinase, provided complete protection from the toxicity of melanin precursors (39). Cells that do not have the enzyme tyrosinase also show a much lower level of toxicity from these precursors indicating a selectivity for melanin synthesizing cells. A question still remains as to how these melanin precursors might be

incorporated into melanoma cells where they can be metabolized into their cytotoxic derivatives.

1.7 GENERAL INTRODUCTION TO AMINO ACID TRANSPORT

Amino acids are the building blocks for all cellular proteins and can be used for energy metabolism. The requirements of the cell for these amino acids are met from two sources: a) extracellular amino acids taken up into the cells and, b) intracellular biosynthesis of certain of the amino acids. In order to obtain the extracellular amino acids, several transport systems exist in animal cells (Table 1.1) (43-46). Almost all cells possess these membrane transport systems for amino acids in order to accommodate a diverse range of intracellular biochemical requirements. The presence and activity of these transporters can be affected by many factors that influence the growth and differentiation of the particular cell type involved (47).

Studies on the transport of nutrients into tissue culture cells have increased since it was proposed that growth rate and malignancy of tumor cells may, to some extent, be controlled by the transport activity of cell membranes (48). A study of the existing transport systems is a necessary first step in examining the regulation of amino acid transport and in determining to what extent this can affect or be affected by cellular

function. In melanoma cells it has been suggested that the increase in melanin synthesis during the melanotic stage is associated with increased activity of amino acid transport as well as increased tyrosinase activity (49).

As a result of transport studies in various cell types and for a variety of amino acids and analogs, a classification system has been developed with which to compare and categorize these transport systems (Table 1.1) (43-46). These amino acid transport systems have been characterized by two principal methods: a) the study of mutants with transport deficiencies, and b) direct kinetic analysis of amino acid fluxes. In mammalian cells the direct analysis approach has been favored, although difficulties can arise when using transported substrates as inhibitors or when different transport systems show overlapping specificities for particular amino acids (32).

In general, transport is dependent on many factors including cell type, proliferation rate, general metabolic activity, and cell specific metabolic activity (44,47). Therefore, it is important to strictly control the conditions under which amino acid transport will be studied in order to clearly interpret the results. The utilization of an <u>in vitro</u> model has made this much simpler since all of the parameters for cell growth can be carefully controlled.

Neutral amino acids such as tyrosine, tryptophan, phenylalanine, alanine and leucine have had their transport mechanism studied in many different cell types (47,50-59). These transport systems have been grouped according to various parameters including substrate specificity and sodium ion dependence (Table 1.1). Three ubiquitous transport systems for neutral amino acids have been characterized; L, A, ASC, as well as the cell specific transport systems T, $B^{o,+}$, $b^{o,+}$ and asc.

System L is a widely distributed transport system that is capable of transporting many different amino acids with different structures. This Na^+-independent transport system was first described for the transport of leucine, but is also capable of accommodating amino acids containing aromatic rings (50,60). System L has a characteristic substrate, 2-aminobicyclo-(2.2.1)-heptane-2-carboxylic acid (BCH), that has been found to be specific for only this transport system. This can be very useful when trying to elucidate the mechanism of transport of a compound.

A variant of the L transport system has been described for mammalian red blood cells (61,62) and rat hepatocytes (63). System T is specific for transporting neutral amino acids that possess aromatic rings such as tyrosine, tryptophan and phenylalanine (61). It has been characterized as a Na^+-independent transport system, with

tryptophan serving as its model substrate. In all cell types investigated except mammalian red blood cells and rat hepatocytes, the substrate specificity of system T completely overlaps that of system L resulting in the identification of only a single transport system for the larger neutral amino acids.

Amino acids with shorter side chains have been found to be transported by system A or ASC. Much study has been done on system A (alanine-prefering) transport since it has been discovered to be important in the liver for the alanine-glucose cycle (45) and identified as a possible target for oncogenic action in neoplastic cells (64). System A is most effective for small neutral amino acids such as alanine, glycine, serine and the analog 2-aminoisobutyric acid (AIB) (44,48). This transport system is Na^+-dependent, has as its model substrate 2-methylaminoisobutyric acid (MeAIB), and is sensitive to hormonal stimulation (48).

A second Na^+-dependent neutral amino acid transport system has been identified. This system which primarily transports alanine, serine, and cysteine is designated ASC. System ASC has a narrower range than system A as to the length of the amino acid side chains it finds acceptable (45,65). In addition, system ASC can be distinguished from system A on the basis of its intolerance of N-methylated substrates (eg. MeAIB) (44).

A Na^+-independent version of this transporter has been characterized in human red blood cells and has been designated system asc (62). With the exception of the Na^+-dependence, both ASC and asc have similar substrate characteristics and kinetics.

More recently, Van Winkle et al (58,59) have described the presence of two additional processes that are capable of transporting bulky zwitterionic or cationic amino acids. $B^{o,+}$ designates the Na^+-dependent form, and $b^{o,+}$ the Na^+-independent. So far, these transport mechanisms have only been identified in the preimplantation mouse conceptuses model.

1.8 AMINO ACID TRANSPORT AND TOXICITY

The relationship between cellular transport and cytotoxicity has been studied for various chemotherapeutic agents. One amino acid analog of interest, the L-phenylalanine mustard melphalan, is used clinically in the treatment of multiple myeloma (66) and malignant melanoma (67). The transport of this compound into various cell types has been studied and its effects as a chemotherapeutic agent have been linked, at least partially, to its transport (66,68,69).

The mechanism of transport of melphalan has been found to be cell-type dependent. When investigating L5178Y murine leukemia lymphoblasts, melphalan uptake was

found by Begleiter et al (66) to be strongly inhibited by the leucine analog BCH, but not by the System A specific substrate MeAIB or the System ASC substrate AIB. The transport was not affected by the addition of alkylating agents, while amino acid analogs caused a decrease in the melphalan transport (64). Therefore, melphalan transport closely resembles amino acid transport, potentially useful information about its interaction with the cells.

In other murine leukemia cells melphalan appears to be transported by two separate neutral amino acid transport systems, one of which is a BCH sensitive, sodium-independent L system and the other a BCH sensitive, sodium dependent system (68).

Melphalan is transported by the murine leukemia L1210 cell through carriers of the L-type (69). The reduction in cytotoxicity by single amino acids correlated with a reduction in drug uptake. Additional studies indicated that melphalan transport is mediated through a similar system by cells maintained in vivo and that administration of leucine with melphalan negated the latter's therapeutic efficacy. Studying the cytotoxic effects in relation to the inhibition of amino acid transport allowed for a partial explanation of the action of this compound.

With melanoma cells the uptake of a tyrosine homolog, 4-S-CP has been partially studied (Figure 1.3)

(26,70). 4-S-CP was found to be specifically incorporated into the melanotic melanoma cells (HMV II) which are engaged in active melanin synthesis, but was scarcely incorporated into HeLa S3 or HMV-I cells which have no activity for melanin synthesis (70). In this system, active tyrosinase is required for the incorporation of 4-S-CP. There is evidence that 4-S-CP and related compounds are effective against malignant melanoma (26-33). Therefore, there may exist a relationship between the incorporation of these compounds into melanoma cells and the observed cytotoxicity similar to that found for the amino acid analog melphalan. However, it is still unclear how and why tyrosine and the tyrosine homolog 4-S-CP are selectively transported into melanoma cells. This study characterizes the transport of tyrosine, in vitro, for the human melanotic melanoma cell line, SK-MEL 23.

Table 1.1

General amino acid transport systems in animal cells

Na^+-dependent	Substrates
ASC	alanine, serine, cysteine, AIB
GLY	glycine, serine, sarcosine
N	glutamine, asparagine
A	alanine, glycine, proline, AIB, MeAIB, methionine
$B^{o,+}$	cationic, bulky amino acids

Na^+-independent	Substrates
L	leucine, phenylalanine, alanine, valine, tyrosine, tryptophan, BCH
T	tryptophan, tyrosine, phenylalanine
asc	alanine, serine, cysteine, AIB
C	alanine, cysteine
$b^{o,+}$	cationic, bulky amino acids

Cationic	Substrates
y^+ (Na^+-independent)	diamino acids, arginine, cationic form of histidine

Anionic	Substrates
X^- (Na^+-dependent)	aspartate, glutamate
x^- (Na^+-independent)	aspartate, glutamate

Figure 1.1

Melanocyte structure

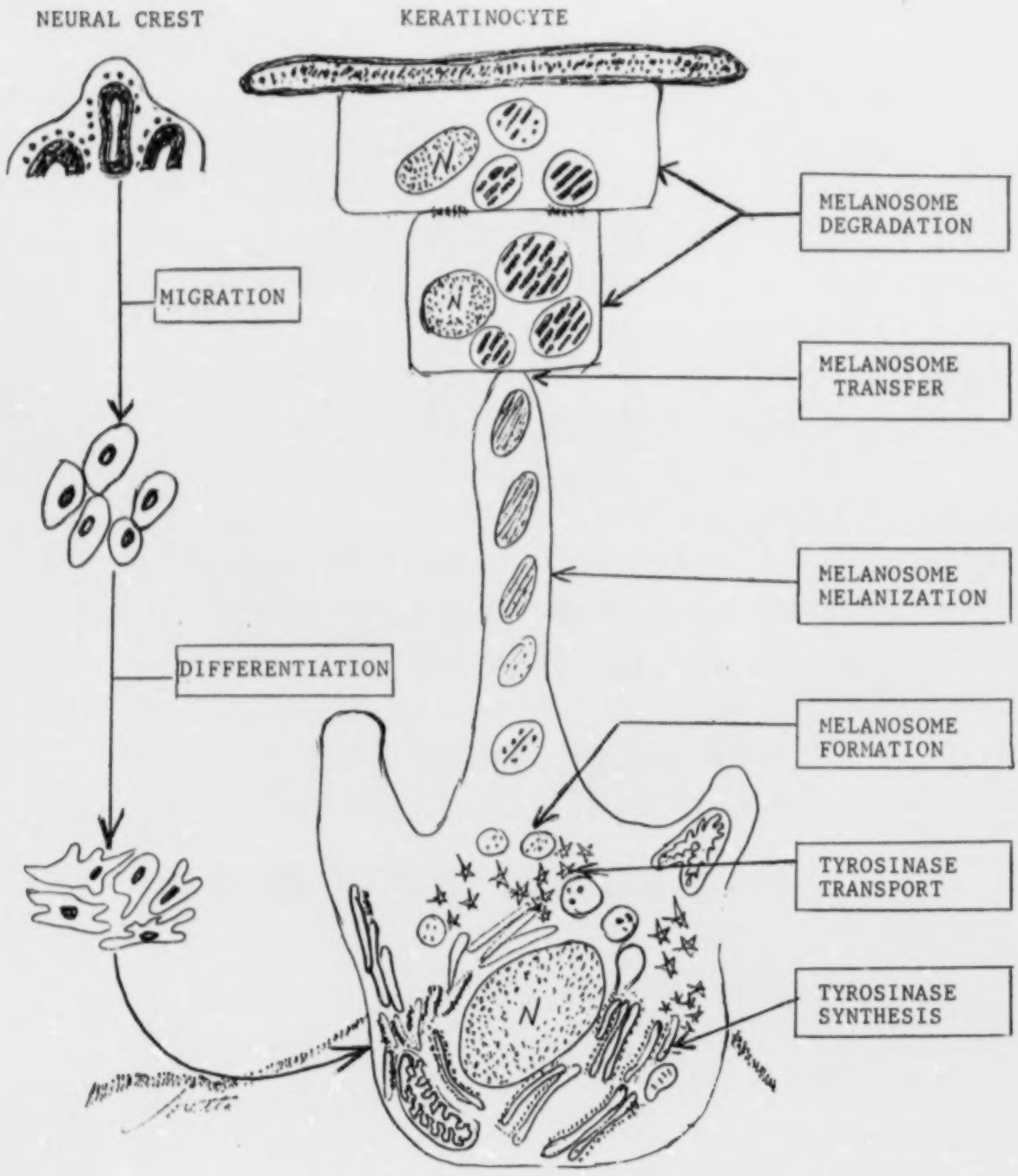


Figure 1.2

Melanin synthetic pathway

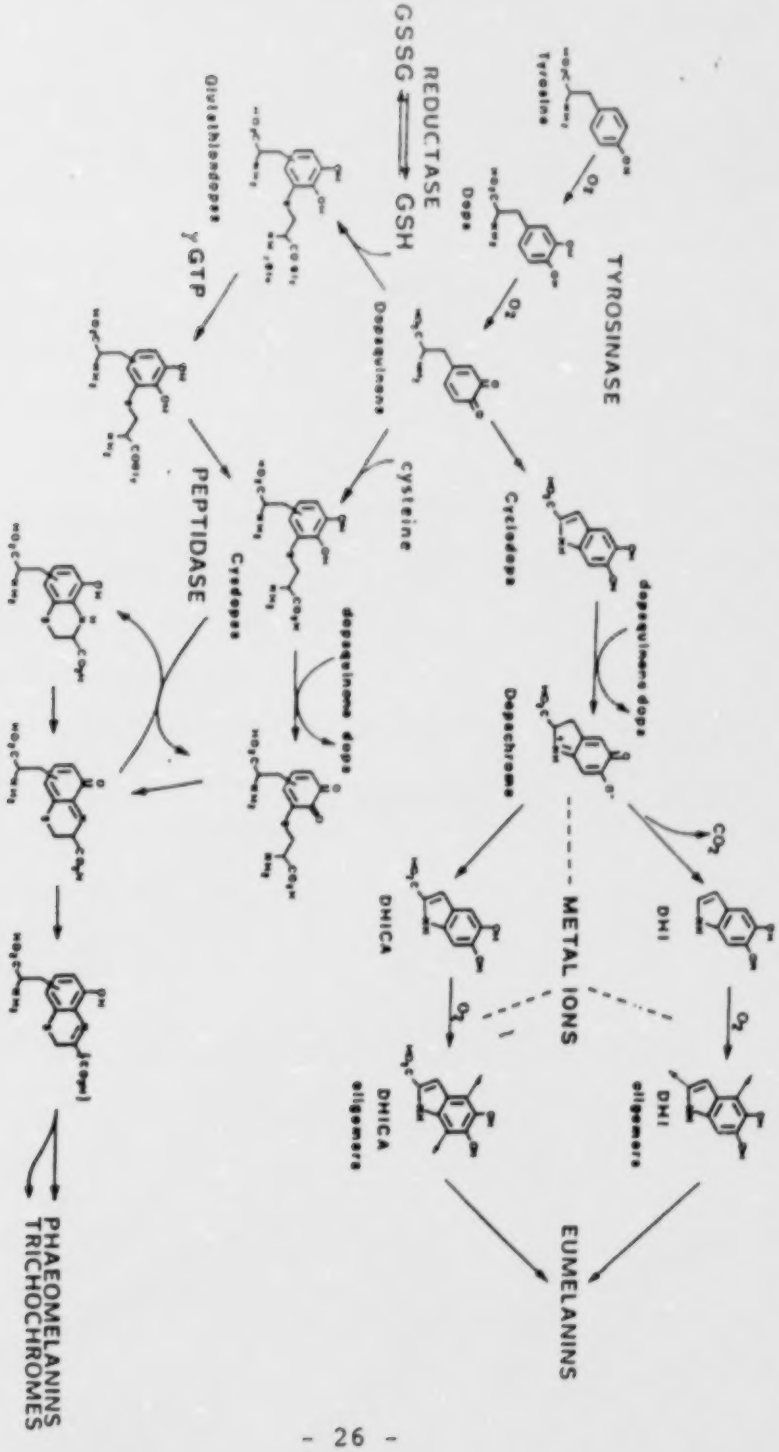
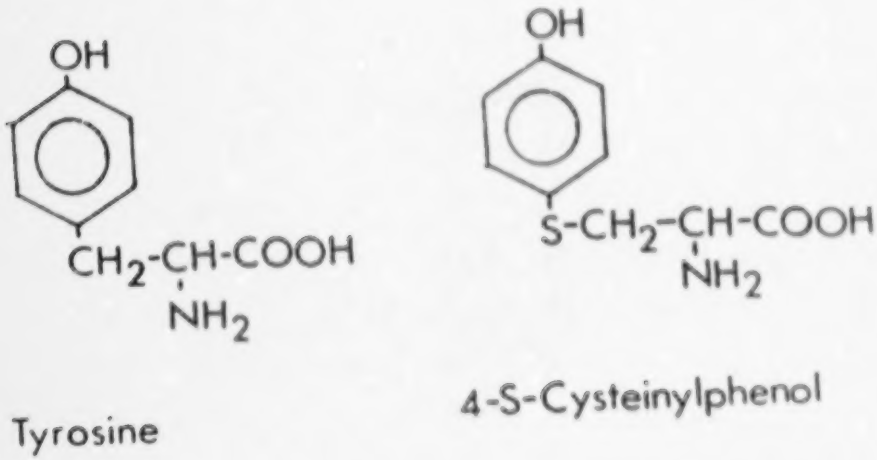


Figure 1.3

Chemical structure of the amino acid tyrosine and its sulfur homolog 4-S-cysteinylphenol (4-S-CP)



Tyrosine

4-S-Cysteinylphenol

CHAPTER 2
MATERIALS AND METHODS

2.1 MATERIALS

Minimal essential medium (MEM), fetal bovine serum (FBS), trypsin-EDTA, and penicillin-streptomycin were purchased from Gibco (Grand Island, NY). The 24-well plates and T-150 flasks used for culturing the cells were obtained from Corning (Corning, NY). L-[ring-3,5-^{3}H]-tyrosine was obtained from NEN (Mississauga, Ontario) at a specific activity of 46.7 Ci/mmol and stored at 4° C. The system L specific substrate, BCH, was obtained from Calbiochem (San Diego, CA). The other inhibitors and chemicals used were of the highest quality available and were obtained from Sigma Chemical Co. (St. Louis, MO).

4-S-CP was synthesized by Dr. S. Ito (Fujita Gakuen University, Toyoake, Japan), the synthesis being reported previously (27,71). Briefly, a mixture of phenol and L-cystine was refluxed in HBr for 2 hours. The resulting orange solution was evaporated to dryness and the residue dissolved in water. The acidity of the solution was adjusted to pH 5 and allowed to stand at 4° C for one week. Colorless 4-S-CP precipitated and was collected.

2.2 EQUIPMENT

Liquid scintillation counting was performed using the xylene based scintillation cocktail Aquasol-2 (NEN, Mississauga, Ontario) in a Beckman LS 3801 liquid scintillation counter. For counting the tritiated tyrosine on this equipment, the following settings were used: 10 minute counts, Beckman external standard method of quench correction (H#), a channel setting of 0-400, and automatic quench compensation to maximize the counting efficiency. Results were programmed to calculate disintegrations per minute (dpm) based on a quench correction curve and converted to nmol tyrosine using equation 2.1.

Equation 2.1

$$(\text{nmol tyrosine})_i = \frac{(\text{nmol tyrosine})_t (\text{sample dpm} - \text{blank dpm})}{(\text{total dpm} - \text{blank dpm})}$$

Where;

$(\text{nmol tyrosine})_i$ = represents the internalized amount of tyrosine

$(\text{nmol tyrosine})_t$ = represents the total tyrosine in the uptake medium

sample dpm = the disintegrations per minute from the experimental sample

blank dpm = the disintegrations per minute from the wells subjected to identical experimental conditions, but in the absence of cells

total dpm = the disintegrations per minute in the uptake medium used in the experiment

2.3 PROTEIN ASSAY

The protein content was determined using the Bradford dye-binding assay available in kit form from Bio-Rad (72). This assay relies on the differential color change of the assay dye, detectable at 595 nm, in the presence of various concentrations of protein.

The assay involved the mixing of 0.1 mL of sample or standard with 5 mL of 1:4 diluted dye reagent. The solution was gently mixed and after a period of five minutes to one hour, the absorbance at 595 nm was determined. The reagent blank consisted of sample solution with no protein and protein dye.

The color change was detected on a Beckman DU-65 spectrophotometer and then compared against a standard curve prepared using bovine serum albumin (BSA) as the standard (Figure 2.1).

2.4 CELL CULTURE

The human melanotic melanoma cell line, SK-MEL 23, was the subject of investigation in these studies. This cell line was kindly supplied by Dr. A. N. Houghton of the Memorial Sloan-Kettering Cancer Centre (NY). Originally, this adherent cell line was derived from a metastatic lesion of human melanoma and has been characterized as having both a high tyrosinase level and being highly pigmented (73).

A frozen stock of SK-MEL 23 cells kept in liquid nitrogen and aliquots were defrosted and grown when required. Cells were tested for the characteristic high tyrosinase activity before use.

These cells were cultured in a Steri-cult 200 incubator (Forma Scientific) under the following standard conditions: 37° C, 5% CO_2, and 95% humidity. The culture medium was MEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 ug/mL streptomycin. After 2-3 days of growth in 150 cm^2 tissue culture flasks, the subconfluent cells were collected by trypsinization and transferred to 24 well plates where the transport assays were performed.

2.5 TYROSINASE ASSAY

One characteristic that is unique to this cell line is the tyrosinase activity. Each frozen stock was thawed and assayed for tyrosinase activity before use in the transport studies.

The tyrosinase assay, as modified by Townsend et al (74), is based upon the enzymatic conversion of L-[3,5-^{3}H]tyrosine to dopa with the release of ^3H_2O in the process.

The assay is initiated by the addition of reaction mixture consisting of 21,000 dpm L-[3,5-^{3}H]tyrosine, 1.5 mM tyrosine, 0.6 mM dopa, TES/TRIS pH adjusting buffer

(pH 7.8) and 0.025 mL sample. The tubes are incubated at 37°C for 3 hours and the reaction stopped by the addition of 0.5 mL of 1% charcoal. The tubes are mixed and incubated at 0°C for 15 minutes and centrifuged at 1000g for 10 minutes. A 0.4 mL aliquot is transferred to a Dowex 50Wx12 200-400 mesh column. The column is then washed with 0.8 mL of 0.1 N citric acid. The eluent is collected for liquid scintillation counting and the tyrosinase activity determined based upon the ^3H_2O radioactive counts.

The tyrosinase activity varies with the time of incubation (Table 2.1). The highest levels were observed 1 or 2 days after thawing and incubating under the standard cell culture conditions. Beyond two days the tyrosinase activity decreases. This decrease is probably due to the increase in the number of cells in the culture flasks. Once the cells are sub-cultured, the level of tyrosinase activity returns to the previously high level.

2.6 MEASUREMENT OF TYROSINE TRANSPORT

Tyrosine transport was measured using a monolayer-cell washing technique (75,76). To obtain the cell monolayer, SK-MEL 23 cells were cultured in 24 well plates under the standard condition. Initially, the cells were seeded at a density of 4 X 10^4 per well in MEM medium and allowed to grow for 2-3 days prior to the

assay procedure being performed. These conditions resulted in 70-80% confluence for the assay procedure.

To prepare the cells for the assay procedure, the monolayer was washed 3 times with a transport buffer (pH 7.4) consisting of 140 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.9 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 25 mM HEPES (328 mOs). The cells were then preincubated in this transport buffer for 30 minutes prior to the initiation of the transport assay.

The transport assay was initiated by the addition of the uptake medium made by supplementing the transport buffer with 1 uCi/mL L-[ring-3,5-^{3}H]-tyrosine, unlabeled tyrosine to the desired concentration and, when appropriate, with unlabeled inhibitors. The cells were allowed to incubate with the uptake medium at room temperature for specific periods of time depending on the experimental conditions. Transport was terminated by aspiration of the uptake media and rapidly washing with three 1.0 mL washes of ice-cold transport buffer. The total wash sequence was completed in less than 20 seconds. One mL of 0.2 N NaOH was added to each well and allowed to stand overnight to dissolve the cells. From this a 0.1 mL sample was taken to determine radioactivity by liquid scintillation counting. A second sample was taken and assayed for protein content by the Bradford dye-binding method using BSA as the standard (72).

Table 2.1

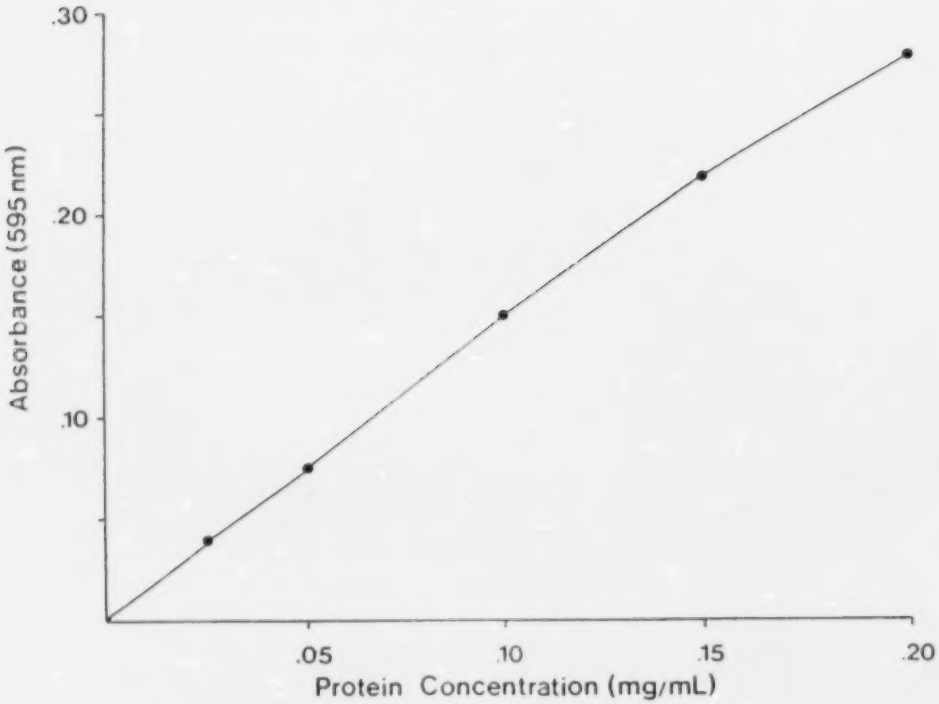
Tyrosinase activity of SK-MEL 23 cultured cells

	Tyrosinase activity (nmol/hr/10^6 cells)
Day 1	4.1 ± 0.1
Day 2	5.2 ± 0.4
Day 3	2.8 ± 0.2
Day 4	0.2 ± 0.01

Tyrosinase activity was determined for cultured SK-MEL 23 cells under standard cell culture conditions after thawing frozen stocks. L-[3,5-^{3}H]tyrosine is enzymatically converted to dopa and ^3H_2O, which is then counted by a liquid scintillation counter. Values are means ± SE of triplicate determinations.

Figure 2.1

Typical standard curve used in the determination of protein content by the Bradford assay with bovine serum albumin (BSA) as the standard.



The Bradford assay for protein determination relies on the detection of a color change at 595 nm in response to the addition of protein to the assay dye. The protein assay consisted of 0.1 mL of sample or standard added to 5 mL of 1:4 diluted dye reagent. After mixing, the solution was allowed to stand for five minutes to one hour. This color change is detected on a spectrophotometer and compared to the standard curve to obtain the experimental value. Values are means ± SE of triplicate determinations. The lack of error bars represents errors so small that they fall within the points.

CHAPTER 3
RESULTS

3.1 TIME COURSE OF TYROSINE TRANSPORT

The SK-MEL 23 human melanoma cells were washed and preincubated in the amino acid free transport buffer for 30 minutes prior to the initiation of the transport assay. This would reduce the influence of intracellular stores of amino acids on the transport of tyrosine (75,76). Following the preincubation period, a time course of tyrosine uptake was followed by incubating the cells for up to 60 seconds in an uptake medium containing ^{3}H-tyrosine and a final tyrosine concentration of 0.1 mM. The amount of tyrosine taken up into the cells was determined using a liquid scintillation counter and Equation 2.1. The amount of protein present per well was determined using the Bradford assay. The uptake can be seen to reach a plateau at 60 seconds (Figure 3.1). There was no difference when Na^{+} was removed from the transport buffer and replaced with K^{+}. All subsequent experiments used an incubation time of 20 seconds to approximate initial rates of transport.

3.2 KINETICS OF TYROSINE TRANSPORT

Kinetics is the study of the rates of movement - in the present context, the rate of movement of a transported substrate across a biological membrane. To understand the kinetics of transport is an attempt to understand the interaction between the substrate and the transporter. Two common values used to describe this interaction are the K_m and V_{max} values. K_m is the kinetic constant that represents the affinity of the substrate for the transporter expressed in concentration units. The K_m is the concentration at half maximal transport velocity. There exists an inverse relationship between the K_m value and substrate affinity, that is the lower the K_m value, the higher the substrate-transporter affinity.

The V_{max} value is the maximal velocity of the transport expressed as units of substrate per unit time period per unit of cell number. The V_{max} occurs when the transport system is saturated with substrate. Under constant cell culture conditions, the K_m and V_{max} values should remain relatively constant.

When the conditions of cell growth are altered, the K_m and V_{max} values may change depending on the cellular response to the change. There are four possible scenarios for the transporters in response to these changes: 1) K_m constant, V_{max} constant, 2) K_m changes,

V_{max} constant, 3) K_m constant, V_{max} changes, and 4) K_m changes, V_{max} changes. K_m values will vary if the nature or type of transporter is altered in the response. In this case V_{max} values may or may not change. If the V_{max} value changes, but the K_m remains constant, this indicates a change in the number of transporters involved, or an increase in the turnover of the transporter.

Transport kinetics are studied by observing the transport characteristics at various substrate concentrations. The relationship between substrate concentration and transport velocity can then be interpreted using various mathematical or graphical methods to obtain the values of interest.

The Michaelis-Menton equation also known as the basic velocity equation or ligand binding equation has many useful interpretations in the fields of enzyme and transport kinetics (77,78).

Equation 3.1

Michaelis-Menton Equation

$$V = V_{max} \frac{[S]}{[S] + K_m}$$

Where;

V = rate of transport of species into the cell

[S] = concentration of species outside of the cell

K_m = concentration at half maximal transport across the membrane

V_{max} = the rate of transport if the transport system is saturated with substrate

Since a plot of the Michaelis-Menton equation (V vs [S]) yields a hyperbola, it is difficult to determine V_{max} and the substrate concentration that yields half of the V_{max} value ie., K_m.

To facilitate the determination of the kinetic constants, the data are usually plotted in a linear form. The Lineweaver-Burk reciprocal plot is the most widely used diagnostic plot.

Equation 3.2

Lineweaver-Burk Equation

$$\frac{1}{V} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}$$

Where;

V = rate of transport of the species into the cell

[S] = concentration of species outside the cell

K_m = concentration at half maximal transport across the membrane

V_{max} = the rate of transport if the transport system is saturated with substrate

The plot of 1/V vs 1/[S] is linear with;

slope = K_m/V_{max}

y-intercept = $1/V_{max}$

x-intercept = $-1/K_m$

Care must be taken to choose substrate concentrations in the neighborhood of the K_m value otherwise the graph will be too horizontal or too vertical to allow for proper interpretation of the intercepts. The use of the Lineweaver-Burk plot in the evaluation of kinetic data has been criticized for two major reasons: 1) equal increments of [S] that yield equally spaced points on the basis of the V vs [S] plot do not yield equally spaced points on the reciprocal plot and more importantly, 2) small errors in the determination of V are magnified when reciprocals are taken. This means that one or two "bad" points can introduce considerable error to the slope of this plot.

Other linear transformations of the basic equation have been derived and under certain circumstances may be more suitable and yield more reliable estimates of the kinetic constants.

The Woolf-Augustinsson-Hofstee plot of V vs V/[S] does not involve reciprocals and as a result may be more reliable when the error in V is significant.

Equation 3.3

Woolf-Augustinsson-Hofstee Equation

$$V = -K_m \frac{V}{[S]} + V_{max}$$

Where;

K_m = concentration at half maximal transport across the membrane

V = rate of transport of the species into the cell

[S] = concentration of species outside of the cell

V_{max} = the rate of transport if the transport system is saturated with substrate

The plot of V vs V/[S] is linear with;

slope = $-K_m$

y-intercept = V_{max}

x-intercept = V_{max}/K_m

Since no reciprocals are required, this eliminates the errors that occur in their determination.

The kinetics of tyrosine transport in the SK-MEL 23 cell line was studied using the monolayer cell washing technique at tyrosine concentrations ranging from 0.05-0.8 mM. The tyrosine uptake was determined by liquid scintillation counting and protein content by the Bradford assay. When the transport velocity at each of these concentrations is graphed as a function of tyrosine concentration, a typical saturation curve is produced (Figure 3.2).

Mathematical manipulation of the basic equation allows us to use either the Lineweaver-Burk plot of 1/V vs 1/[S] (Figure 3.3), or the Woolf-Augustinsson-Hofstee plot of V vs V/[S] (Figure 3.4) to graphically determine the K_m and V_{max} values for tyrosine transport. When

graphed, both of these linear transformations of the Michaelis-Menton equation resulted in the determination of similar K_m and V_{max} values. For the Lineweaver-Burk plot, the K_m is the negative inverse of the X-intercept and the V_{max} the inverse of the Y-intercept (K_m = 0.164 mM, V_{max} = 21.4 nmol/min/mg protein). Because of the potential problems with the error when performing the Lineweaver-Burk transformation, the Woolf-Augustinsson-Hofstee plot, considered more reliable, was used for the final determination of the kinetic parameters. From this linear graph the K_m is represented by the negative slope, and the V_{max} by the y-intercept. Based on the best fit line determined statistically using the SPSS linear regression program, the K_m was calculated to be 0.164 $\pm$ 0.016 mM and the V_{max} to be 21.6 $\pm$ 1.1 nmol/min/mg protein (mean $\pm$ SE, n=3).

3.3 INHIBITION ANALYSIS

The use of inhibitors can be very useful in identifying the nature of amino acid transport. Certain amino acids and their analogs have been shown to be specific for one of the previously described transport systems (Table 1.1). The nomenclature used to describe the different systems reflects the specific nature of some of these compounds (eg. system L for leucine, system

T for tryptophan, and system ASC for alanine, serine, and cysteine).

If any of these specific substrates can be shown to inhibit the transport of tyrosine into the cells, then this would be a good indication that similar transport mechanisms are involved. By using a number of different inhibitors, the nature of the transport of the substrate in question can be determined.

Using the monolayer-cell wash procedure, the inhibition of tyrosine transport into SK-MEL 23 cells was studied in the presence of various amino acids and their analogs. Inhibition analysis was performed using a tyrosine concentration of 0.1 mM and an inhibitor concentration of 1.0 mM in the uptake medium. The transport of radiolabeled tyrosine was determined by liquid scintillation counting and protein content by the Bradford assay.

Of the compounds tested as tyrosine transport inhibitors, the most significant inhibition came from the aromatic amino acids, tryptophan (78.1%) and phenylalanine (77.1%), and from the branched side chain amino acid leucine (66.6%) (Table 3.1). The leucine analog, BCH, a specific substrate of system L transporter, and the sulfur homolog of tyrosine, 4-S-CP, were also very effective inhibitors of tyrosine transport with percent inhibition being 63.0% and 61.2%

respectively. The remaining compounds had little or no effect on the transport of tyrosine in this cell line with percent inhibition being less than 30% in each case. The absence of substantial inhibition with the cationic amino acid arginine (14.3%) rules out any involvement of the recently described neutral and cationic amino acid transporters $B^{0,+}$ and $b^{0,+}$.

These experiments were repeated in the absence of Na^+ in the uptake medium by substituting KCl for NaCl producing a K^+ medium. The inhibitors that produced the greatest inhibition in the presence of Na^+, also showed the greatest inhibition in the K^+ medium. Those values did not differ substantially between the two groups.

The inhibitors that were in the group with less than 30% inhibition in the presence of Na^+ again accounted for less than 30% inhibition in the K^+ medium.

3.4 SODIUM DEPENDENCE OF TYROSINE TRANSPORT

There are two major categories of amino acid transport systems that have been identified, Na^+-dependent and Na^+-independent (Table 1.1). To directly compare the transport system for tyrosine in SK-MEL 23 human melanoma cells in the presence or absence of Na^+, the transport was studied at two different tyrosine concentrations. The Na^+ was replaced in the K^+ uptake medium by substituting KCl for NaCl. The monolayer-cell

wash technique was used with an incubation time of 20 seconds to approximate the initial rate of transport. Tyrosine uptake was determined by liquid scintillation counting and protein content by the Bradford assay. At both the 0.1 mM and 0.6 mM tyrosine concentrations, there was no statistical difference in the observed transport (Table 3.2). This result is in agreement with the time course and inhibition analysis where the presence or absence of Na^+ in the uptake medium had no measurable effect.

3.5 SEQUENTIAL INHIBITOR ANALYSIS

From the initial inhibition analysis, it is apparent that there are several amino acids and their analogs that are very good inhibitors of tyrosine transport. These include phenylalanine, tryptophan, leucine, the leucine analog BCH, and the S-homolog of tyrosine, 4-S-CP. Of these effective inhibitors, tryptophan and BCH were chosen for further analysis because each has been associated with a specific amino acid transport system in other cell types (48,61,62). Tryptophan is the characteristic substrate for system T and BCH the specific substrate for system L. The tyrosine homolog, 4-S-CP, was also further analyzed because of its close structural similarity to tyrosine (Figure 1.3) and its relatively high inhibition of tyrosine transport (61.2%).

First, tryptophan or BCH was added alone to 0.1 mM tyrosine uptake medium at concentrations ranging from 0-9 mM. The uptake media was incubated with SK-MEL 23 cells for 20 seconds, and using the monolayer-cell wash procedure, the tyrosine transport was determined. The tyrosine uptake was determined by liquid scintillation counting and protein content by the Bradford assay. Transport inhibition was expressed as a percentage of control transport in which cells were incubated with only the tyrosine added to the uptake medium. Both tryptophan and BCH showed a similar effect due to increasing inhibitor concentration on tyrosine transport inhibition (Figure 3.5). There was a rapid increase in the inhibition caused by increasing the concentration of these compounds followed by a plateau phase in which there was no further inhibition observed.

Next, these two inhibitors, along with the synthetic tyrosine homolog, 4-S-CP, were sequentially added at increasing concentrations and the inhibition of tyrosine transport was followed in a similar fashion. Sequential addition of these tyrosine transport inhibitors would allow for the identification of a transport system or systems that may be masked by the presence of a higher capacity transporter or a transporter with overlapping specificities. The SK-MEL 23 cells were incubated with 0.1 mM tyrosine and either: a) increasing concentrations

of BCH, 2.25 mM BCH plus increasing concentrations of 4-S-CP, and 2.25 mM BCH plus 10 mM 4-S-CP plus increasing concentrations of tryptophan added simultaneously, or b) the addition of the same compounds at the same concentrations but reversing the order of addition of BCH and tryptophan (Figure 3.6). Tyrosine uptake was determined by liquid scintillation counting and protein content by the Bradford assay.

The inhibition of tyrosine transport in response to the increasing concentrations of these compounds was similar even when the sequence of addition of the BCH and tryptophan was reversed. That is, there was no further inhibition over and above that which was seen with the single inhibitor alone upon addition of the second or even third inhibitor. Since each of these compounds alone can cause substantial inhibition of tyrosine transport, this would indicate that all of the inhibitors were working in a similar manner to inhibit tyrosine transport.

3.6 DIXON PLOT FOR 4-S-CP

4-S-CP is structurally related to tyrosine (Figure 1.3) and the potential chemotherapeutic effects are of interest. In order to more closely determine the nature of the 4-S-CP inhibition of tyrosine transport, the

inhibition constant (K_i) for 4-S-CP inhibition was determined graphically using the Dixon plot (78).

Equation 3.4

Dixon Equation

$$\frac{1}{V} = \frac{K_m}{V_{max}\ K_i\ [S]}\ [I] + \frac{1}{V_{max}}\ \left(1 + \frac{K_m}{[S]}\right)$$

Where;

V = rate of transport of species into the cell

K_m = concentration at half maximal transport across the membrane

V_{max} = the rate of transport if the transport system is saturated with substrate

K_i = inhibition constant

[S] = concentration of species outside of the cell

[I] = concentration of inhibitor outside of the cell

By plotting 1/V vs [I] at different fixed concentrations of substrate provides a way of identifying the type of inhibition and of determining the K_i. The lower the value of K_i, the greater is the degree of inhibition at any given substrate and inhibitor concentration. K_i is equivalent to the concentration of inhibitor that doubles the slope of the 1/V vs 1/[S] plot.

Using 0.05, 0.1, and 0.2 mM tyrosine concentrations and increasing concentrations (0.05-1.0 mM) of 4-S-CP in the uptake media, the tyrosine transport was followed

utilizing the monolayer-cell wash procedure with an incubation time of 20 seconds at room temperature. Tyrosine uptake was determined by liquid scintillation counting and protein content by the Bradford assay. The results can be graphically expressed to determine the inhibition constant for the effect of 4-S-CP on tyrosine transport, the K_i value (Figure 3.7). The Dixon plot shows that the 4-S-CP acts as a competitive inhibitor of tyrosine transport and the K_i value was found to be 0.3 mM.

3.7 OSMOLARITY EFFECTS

Cells can regulate their volume by the movements of amino acids across the membrane (79). In vitro, this can be simulated by changing the osmolarity of the incubation solution. By varying the concentration of NaCl and not substituting for the loss of those ions, the effect of changing the osmolarity of the uptake medium on tyrosine transport in SK-MEL 23 melanoma cells could be determined. Using the uptake medium containing 0.1 mM tyrosine and the monolayer-cell wash technique, the NaCl concentration was varied from 70-150 mM without compensation for the change in NaCl concentration producing uptake media with 188-328 mOs. The change in NaCl concentration had no effect until the concentration was decreased to 70 mM (Figure 3.8). Even at this low

NaCl concentration, the tyrosine transport was still 78% of the control value. Above the 140 mM NaCl control, the transport was also unchanged. Therefore, there seems to be little osmolarity effects on tyrosine transport even when the NaCl concentration in the uptake media was decreased by half and the osmolarity of the solution was decreased from 328 to 188 mOs.

3.8 MANIPULATION OF TYROSINE TRANSPORT

Tyrosine is required by SK-MEL 23 melanoma cells for both general protein synthesis and for the production of pigment. Changing the cellular requirements for tyrosine by altering the melanin synthesis pathway may affect the transport of this essential amino acid into the melanoma cells. If a regulation mechanism for the transport of tyrosine and tyrosine homologs could be found, then this may be a mechanism to increase the accumulation of cytotoxic tyrosine homologs in melanoma cells.

There are several factors that could contribute to a change in melanogenesis. One is by decreasing the activity of tyrosinase in the melanosome. Phenylthiourea (PTU) has been shown to inhibit tyrosinase, thereby decreasing or eliminating the activity of this melanin producing enzyme (39,80). Conversely, stimulation of tyrosinase activity can occur in the presence of melanocyte stimulating hormone (MSH). This endogenous

hormone causes an increase in pigment production in human melanoma cells (7,11,39). By incubating the SK-MEL 23 cells with these compounds, we can study the effects of changing melanin synthesis on tyrosine transport.

The SK-MEL 23 cells were seeded into the 24 well plates and allowed to attach overnight. The medium was changed from MEM to MEM supplemented with either 0.1 mM PTU or 2 ug/mL MSH. The cells were then incubated under the standard conditions for two days at which time the tyrosine transport assay was performed. All results were compared to a control group that were grown in the presence of MEM alone. Tyrosine uptake was determined by liquid scintillation counting and protein content by the Bradford assay.

The addition of PTU had no effect on the tyrosine transport in this cell line as the PTU treatment group showed no change from the control group (Table 3.3). On the other hand, stimulation of melanogenesis by the addition of MSH to the growth medium increased the transport of tyrosine by 30% compared to the control group.

Table 3.1

Inhibition of tyrosine transport[a] into malignant melanoma cells *in vitro* by amino acids and amino acid analogs

	% Inhibition	
Inhibitor (1.0 mM)	NaCl medium	KCl medium
tryptophan	78.1 ± 1.0	69.2 ± 1.0
phenylalanine	77.1 ± 1.2	76.1 ± 3.1
leucine	66.6 ± 0.8	55.0 ± 2.6
BCH[b]	63.0 ± 0.8	62.3 ± 1.4
4-S-CP[c]	61.2 ± 2.8	63.0 ± 2.4
cysteine	28.2 ± 0.9	0.0
alanine	18.6 ± 1.1	14.1 ± 1.4
cystine	17.7 ± 1.2	27.9 ± 1.6
arginine	14.2 ± 3.6	0.9 ± 0.9
serine	13.5 ± 0.6	3.5 ± 1.9
glutamine	13.3 ± 2.5	3.4 ± 1.7
MeAIB[d]	9.8 ± 1.9	0.0
AIB[e]	5.8 ± 0.6	0.0

a) Tyrosine transport was measured at a 0.1 mM tyrosine concentration in the presence of 1.0 mM inhibitors. K^+ was used as a replacement for Na^+ in the KCl medium experiments. Cells were incubated at room temperature for 20 seconds in the presence of both tyrosine and the inhibitor. The transport was stopped by aspiration of the medium and washing with ice-cold buffer. Values are means ± SE of triplicate determinations.

b) BCH - 2-aminobicyclo-(2.2.1)-heptane-2-carboxylic acid

c) 4-S-CP - 4-S-cysteinylphenol

d) MeAIB - 2-methylaminoisobutyric acid

e) AIB - 2-aminoisobutyric acid

Table 3.2

Comparison of tyrosine transport into melanoma cells *in vitro* in the presence or absence of Na^+ in the uptake medium

Tyrosine Concentration (mM)	Tyrosine Uptake[a] (nmol/mg protein) NaCl medium	KCl medium	t-test (p-value)[b]
0.1	2.49 ± 0.12	2.87 ± 0.09	$p>0.05$
0.6	6.23 ± 0.81	7.02 ± 0.35	$p>0.2$

a) K^+, in the form of KCl, was used as the replacement for Na^+. Cells were incubated for 20 seconds in transport buffer supplemented with either 0.1 or 0.6 mM tyrosine. The cells were washed 3 times with ice-cold buffer then lysed using 0.2 N NaOH and samples taken for determination of [^{3}H]-tyrosine by liquid scintillation and protein content by the Bradford dye-binding assay. Values are means ± SE of at least triplicate determinations.

b) P-values were obtained using Student's t-test.

Table 3.3

Manipulation of tyrosine transport[a] in SK-MEL 23 cells with PTU and MSH

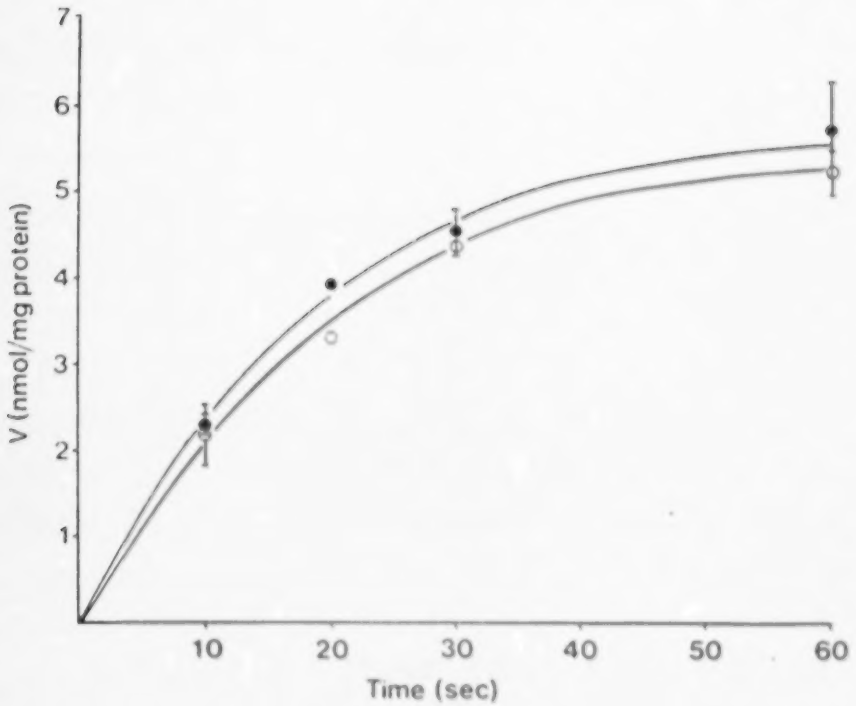
	V (nmol/min/mg protein)	% Control	t-test[b]
Control	6.10 ± 0.02	-	
PTU	6.04 ± 0.44	99	$p>0.5$
MSH	7.92 ± 0.28	130	$p<0.02$

a) Tyrosine transport was measured at a 0.1 mM tyrosine concentration after treatment of the cells for 2 days with either 0.1 mM PTU or 2 ug/mL MSH. Cells were incubated for 20 seconds in the tyrosine supplemented transport buffer. The cells were then washed 3 times with ice-cold buffer then lysed using 0.2 N NaOH and samples taken for determination of [^{3}H]-tyrosine by liquid scintillation and protein content by the Bradford assay. Values are means ± SE of triplicate determinations.

b) P-values were obtained using Student's t-test.

Figure 3.1

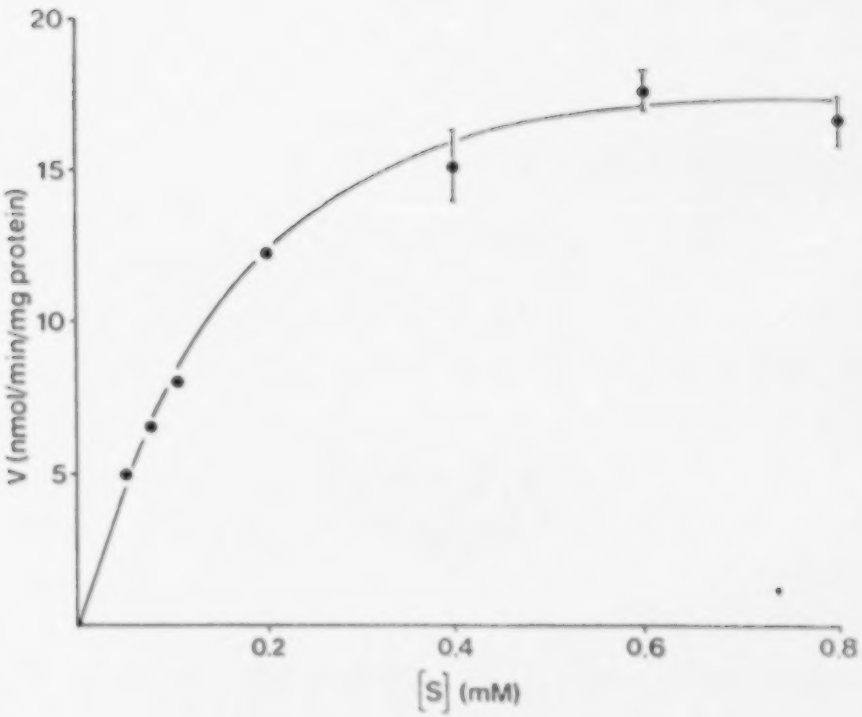
Time course of tyrosine transport in SK-MEL 23 melanoma cells



The time course of tyrosine transport was followed for up to 60 seconds at a tyrosine concentration of 0.1 mM in either NaCl (●) or KCl (○) uptake medium. The cells were then washed 3 times with ice-cold buffer then lysed using 0.2 N NaOH and samples taken for determination of [^{3}H]-tyrosine by liquid scintillation and protein content by the Bradford assay. All subsequent experiments used a 20 second incubation to approximate initial rates of transport. Values are means ± SE of triplicate determinations. The lack of error bars represents errors so small that they fall within the points.

Figure 3.2

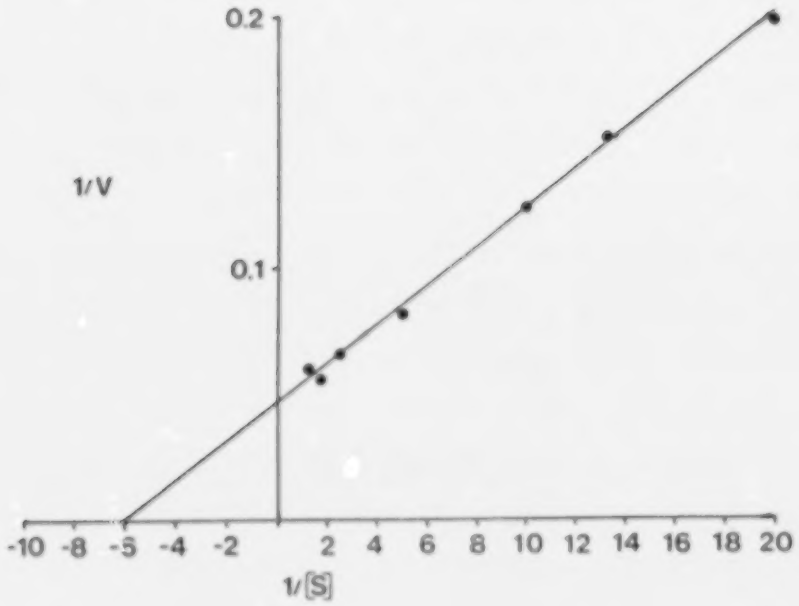
Michaelis-Menton saturation curve of tyrosine transport



Concentration dependence of tyrosine uptake by SK-MEL 23 melanoma cells over the tyrosine concentration range of 0.05-0.8 mM. Transport was measured for 20 seconds in NaCl uptake medium. To stop the transport, cells were washed 3 times with ice-cold buffer and cells lysed using 0.2 N NaOH. Liquid scintillation was used to determine the [^{3}H]-tyrosine uptake and protein content was determined using the Bradford dye binding assay. Values are means ± SE of triplicate determinations. The lack of error bars represents errors so small that they fall within the points.

Figure 3.3

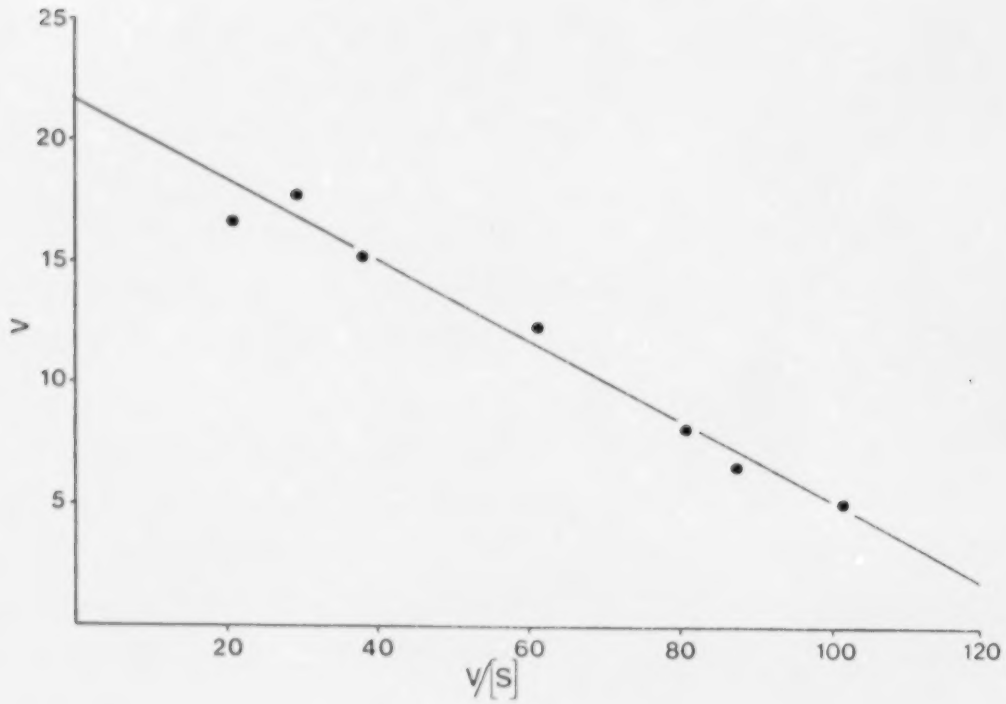
Lineweaver-Burk plot of tyrosine transport data



The Lineweaver-Burk reciprocal plot of 1/V vs 1/[S] can be used to determine K_m and V_{max} values. Tyrosine concentrations from 0.05-0.8 mM were used in the tyrosine transport assay to produce the data for this plot. Cells were incubated for 20 seconds in the tyrosine supplemented transport buffer. The cells were then washed 3 times with ice-cold buffer then lysed using 0.2 N NaOH and samples taken for determination of [^{3}H]-tyrosine by liquid scintillation and protein content by the Bradford assay. Values are means ± SE of triplicate determinations. The lack of error bars represents errors so small that they fall within the points.

Figure 3.4

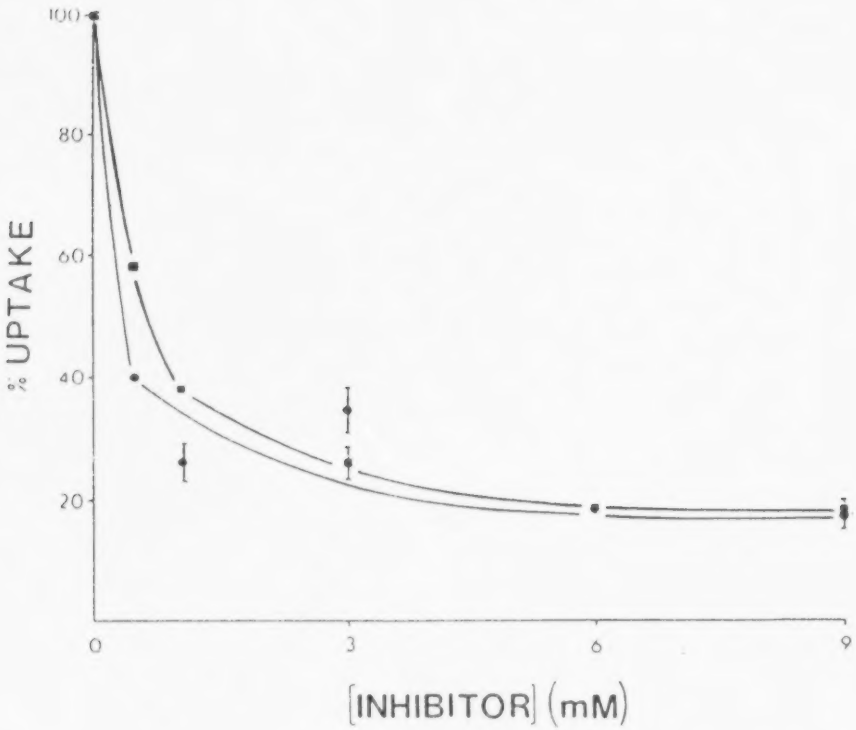
Woolf-Augustinsson-Hofstee plot of tyrosine transport data



Using the Woolf-Augustinsson-Hofstee transformation of the basic Michaelis-Menton equation, a linear relationship is used to determine the K_m and V_{max} values. Tyrosine concentrations ranged from 0.05-0.8 mM and transport was measured for 20 seconds in NaCl uptake medium. The cells were then washed 3 times with ice-cold buffer then lysed using 0.2 N NaOH and samples taken for determination of [^{3}H]-tyrosine by liquid scintillation and protein content by the Bradford assay. Values are means ± SE of triplicate determinations. The best fit line was determined using the SPSS linear regression program. The lack of error bars represents errors so small that they fall within the points.

Figure 3.5

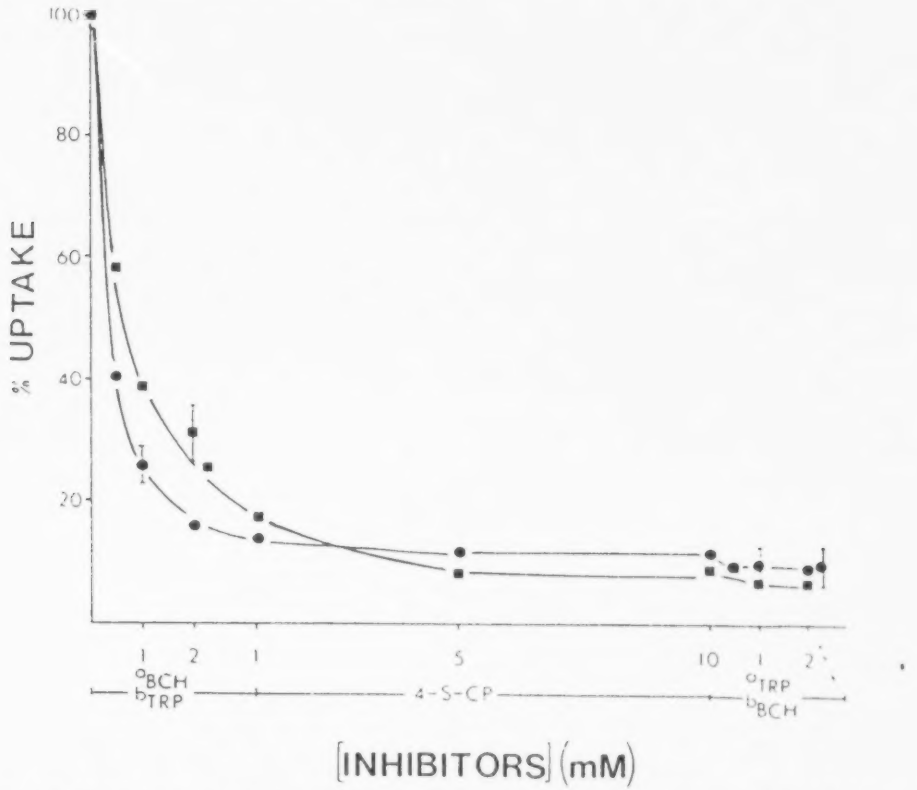
Inhibition of tyrosine transport using either BCH or tryptophan



Tyrosine transport was followed using the NaCl uptake medium supplemented with 0.1 mM tyrosine and 0 - 9 mM BCH or tryptophan as inhibitors. Cells were incubated for 20 seconds in experimental medium. The cells were then washed three times with ice-cold buffer, then lysed using 0.2 N NaOH. Samples were taken for determination of [^{3}H]-tyrosine by liquid scintillation and protein content by the Bradford assay. Control value was 6.82 ± 0.32 nmol/min/mg protein. Values are means ± SE of triplicate determinations. BCH (■); tryptophan (●). The lack of error bars represents errors so small that they fall within the points.

Figure 3.6

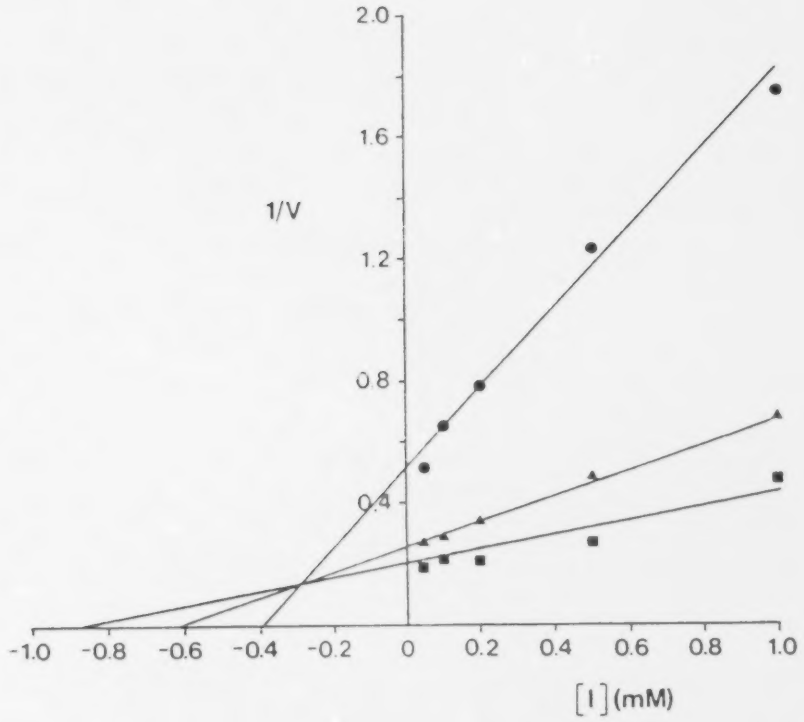
Sequential inhibitor analysis



The effect of BCH, tryptophan, and the sulfur homolog of tyrosine, 4-S-cysteinylphenol (4-S-CP) on tyrosine transport in SK-MEL 23 cells was studied. The SK-MEL 23 cells were incubated with 0.1 mM tyrosine and a) increasing concentrations of BCH, 2.25 mM BCH plus increasing concentrations of 4-S-CP, or 2.25 mM BCH plus 10 mM 4-S-CP plus increasing concentrations of tryptophan added simultaneously (■ ;BCH/4-S-CP/tryptophan). b) Studies the effects of the same compounds on tyrosine transport, but reverses the order of BCH and tryptophan addition (● ; tryptophan/4-S-CP/BCH). Under all conditions the cells were incubated for 20 seconds then washed 3 times with ice-cold buffer. The cells were then lysed using 0.2 N NaOH and samples taken for determination of [^{3}H]-tyrosine by liquid scintillation and protein content by the Bradford assay. Control value was 5.38 ± 0.37 nmol/min/mg protein. Values are means ± SE of triplicate determinations. The lack of error bars represents errors so small that they fall within the points.

Figure 3.7

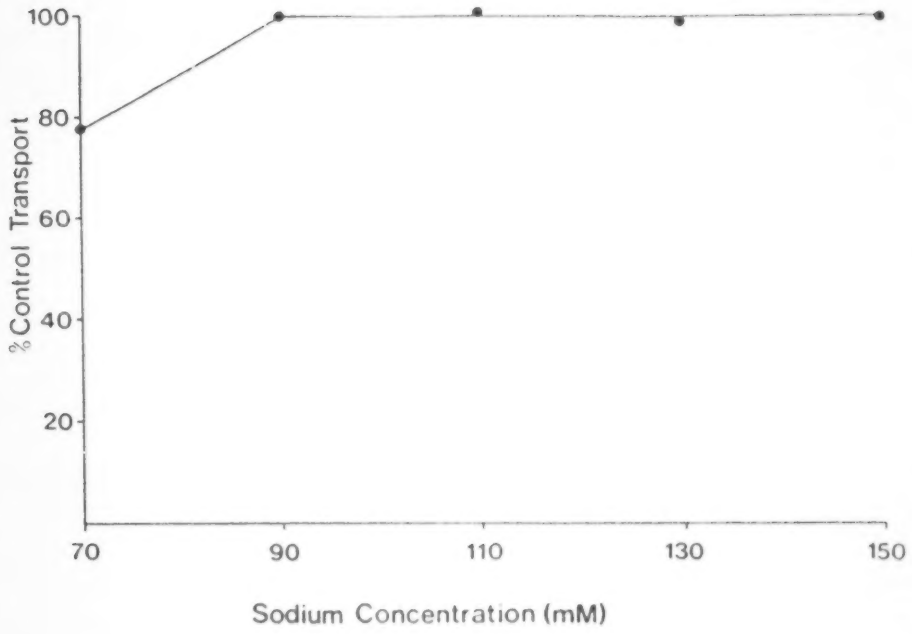
Dixon plot to determine the effects of 4-S-CP on tyrosine transport



A Dixon plot using tyrosine concentrations of 0.05 mM (●), 0.1 mM (▲), and 0.2 mM (■) with 4-S-CP concentrations of 0.05-1.0 mM were used to determine the effect of the addition of this compound on tyrosine transport. Cells were incubated for 20 seconds in the supplemented transport buffer. The cells were then washed 3 times with ice-cold buffer then lysed using 0.2 N NaOH and samples taken for determination of [^{3}H]-tyrosine by liquid scintillation and protein content by the Bradford assay. Values are means ± SE of triplicate determinations. The lack of error bars represents errors so small that they fall within the points. The K_i of this competitive inhibition was determined to be 0.3 mM based on this data.

Figure 3.8

Effects of changing osmolarity of the uptake medium on tyrosine transport



Decreasing concentrations of NaCl without compensation for the loss of those ions produces a hypoosmotic uptake medium. The effects on tyrosine transport were determined at 70-150 mM NaCl concentrations in the presence of 0.1 mM tyrosine. Cells were incubated for 20 seconds in the tyrosine supplemented transport buffer. The cells were then washed 3 times with ice-cold buffer then lysed using 0.2 N NaOH and samples taken for determination of [^{3}H]-tyrosine by liquid scintillation and protein content by the Bradford assay. Control value was 4.29 ± 0.40 nmol/min/mg protein. Values are means ± SE of triplicate determinations. The lack of error bars represents errors so small that they fall within the points.

CHAPTER 4
DISCUSSION

4.1 CHARACTERIZATION OF TYROSINE TRANSPORT

Tyrosine is an amino acid that has a unique function in melanocytes and melanoma cells. In addition to its involvement in general protein synthesis, tyrosine is required for the production of the melanin pigments, eumelanin and pheomelanin. Therefore, a better understanding of the mechanism of tyrosine transport in pigment producing cells will have implications not only for the understanding of melanogenesis, but also for the development of new chemotherapeutic agents based on the melanin synthetic pathway.

The kinetic data from this study, obtained using a monolayer-cell wash assay procedure, indicate that the tyrosine transport in SK-MEL 23 human melanoma cells has a K_m of 0.164 ± 0.016 mM and a V_{max} of 21.6 ± 1.1 nmol/min/mg protein (mean SE). In the three studies where the effects of NaCl and KCl medium can be compared, ie., time course, inhibition, and NaCl replacement, there appears to be little change in the transport characteristics in the absence of Na^+ (Figure 3.1 and Table 3.1,3.2). Therefore, tyrosine transport in this cell line can be

classified into the Na^+-independent category of amino acid transport systems.

Specific inhibitors can be very useful in determining the nature of amino acid transport (43,44,81). If it can be demonstrated that there is a decrease in transport because of the presence of another compound, this compound can be assumed to act on a similar transport system. Thirteen different potential inhibitors were tested for their effects on tyrosine transport. Inhibition with the system A specific substrate MeAIB and the system ASC substrate AIB was minimal (Table 3.1). Both of these transport systems are also Na^+-dependent, negating any direct involvement with tyrosine transport in this cell line because of the Na^+-independent nature of the transport.

Phenylalanine had previously been shown to decrease tyrosine incorporation into melanin by preventing the uptake of tyrosine in RPMI-3460 hamster melanoma cells (82). In this study phenylalanine, which can be transported by either system L or system T, produced 77.1% inhibition of tyrosine transport in the human melanotic melanoma cell line SK-MEL 23.

BCH inhibition is a characteristic feature of system L transport because of its selectivity for only this transporter (44). In this study, the addition of BCH to

the uptake medium caused 63.0% inhibition of tyrosine transport in the SK-MEL 23 melanoma cells.

Tryptophan is the characteristic substrate for system T in erythrocytes (61,62) and hepatocytes (11), but can also be transported by system L in other cell types (47,48). In the SK-MEL 23 melanoma cell line, tryptophan inhibition of tyrosine transport has characteristics similar to those of BCH in every comparable situation (Figure 3.5,3.6 and Table 3.1). Both BCH and tryptophan had similar concentration effects in the single inhibition experiment (Figure 3.5) and in the sequential inhibition experiment (Figure 3.6). System L and system T are both capable of transporting aromatic amino acids and are known to have overlapping specificities (44,61). It appears that the SK-MEL 23 cells may utilize a single transport system for the aromatic amino acids.

The residual flux that remains after maximal inhibition has been reached (Figure 3.6) is probably due to diffusion or involvement of a H^+ dependent transporter (83,84). This residual flux represents less that 10% of the tyrosine transport observed in this cell line.

All of the present findings, ie., substrate specificity, Na^+-independence, inhibitor analysis, and kinetics, indicate that greater than 90% of tyrosine transport into the human melanotic melanoma cell line,

SK-MEL 23, is similar to that previously described as system L. This transport also appears to be relatively unaffected by rather large changes in the osmolarity of the growth medium. Tyrosine transport in B16 murine melanoma cells has recently been studied and found to be similarly transported by a single mechanism, system L (49). A second set of studies by Jara et al (85,86) concludes that both system L and ASC may be involved in tyrosine transport under different conditions in B16/F10 murine melanoma cells. These studies used methodologies and cells lines that differed from those presented here which prevents a direct comparison of all results. However, it is apparent that tyrosine transport into these melanin producing cells is an essential step in the melanogenesis pathway.

4.2 TRANSPORT AND TOXICITY

Malignant melanoma remains one of the most challenging cancers to treat successfully (22). It has been found to be very resistant to radiation and current chemotherapy regimens making most of the conventional treatments ineffective. Several different approaches have been attempted in an effort to develop a new class of chemotherapeutic agents against melanoma. One approach, which has been used with some success in other treatments, is the use of analogs of natural compounds.

Analogs of amino acids have been used as chemotherapeutic agents with the transport of these analogs into various cell types being implicated, at least partially, in their efficacy. One example is the L-phenylalanine mustard, melphalan, used clinically in the treatment of multiple myeloma (66) and malignant melanoma (67). Melphalan transport has been characterized in normal human lymphoblasts and bone marrow cells (68), cells of human breast and ovarian cancer (68), as well as in murine leukemia cells (66,69). Its effectiveness as a cytotoxic agent in these cell lines has been associated with its transport through amino acid transporters, particularly system L and system ASC.

In a similar fashion, the sulfur homolog of tyrosine, 4-S-CP, has been synthesized in an effort to utilize the melanogenic pathway in the treatment of melanoma (27). By using the tyrosine like compound, it may be possible to produce cytotoxic intermediates intracellularly capable of melanocytic destruction. 4-S-CP, along with other tyrosine homologs, was previously shown to be a substrate of the specific melanin producing enzyme tyrosinase and capable of increasing the life span of melanoma-bearing mice and causing growth inhibition of melanoma tissue <u>in vivo</u> (26,27,87,88).

Using [^{3}H]-4-S-CP, Nakamura et al (70) were able to show that this compound can be selectively incorporated into a human melanotic melanoma cell line, HMV II, *in vitro*. Through a whole body autoradiography study, [^{3}H]-4-S-CP was also shown to accumulate in subcutaneous B-16 melanoma tumors grown in C57BL/6J mice (26,28). In this present study, 4-S-CP was a very good inhibitor of tyrosine transport, resulting in 61.2% inhibition at a 1.0 mM concentration. The inhibition was comparable to the inhibition observed with the other aromatic and neutral amino acids in particular the system L specific leucine analog, BCH.

The K_i for the competitive inhibition of 4-S-CP on tyrosine transport was determined to be 0.3 mM based on the Dixon plot (Figure 3.7). These results would indicate that 4-S-CP crosses the membrane in a fashion similar to that of tyrosine, and that manipulation of tyrosine transport may alter the effects of this potential chemotherapeutic agent.

4.3 REGULATION OF TRANSPORT

Animal cells adapt to fluctuations in extracellular amino acid availability or intracellular requirements by regulating the activation of certain transport systems (62). As cells mature, their requirements for various essential nutrients change with the mechanism of

transport of these compounds also being affected. Using a temperature sensitive Chinese hamster ovary mutant cell line, Shotwell et al was able to show adaptive changes in system L transport (44,89,90). When these cells were grown at less than optimal conditions, the transport activity for leucine increased 2-3 fold. This transport enhancement was reflected by an increase in the V_{max} of leucine uptake with no change in its K_m suggesting an increase in the number of system L transport carriers. The transport enhancement could be prevented by the addition of the protein synthesis inhibitor, cycloheximide, but not by actinomycin D, an inhibitor of transcription (90).

Lobaton et al (91), using a Chinese hamster-human hybrid temperature sensitive cell line was able to demonstrate similar changes in system L transport. Chromosome analysis led to the conclusion that the high leucine transport phenotype was correlated with the loss of human chromosome 20 in this hybrid cell line.

Control by hormones is one mechanism by which nutrient flows are coordinated in multicellular organisms. Amino acids are nutrients whose levels are affected by the addition of hormones and other compounds that alter cellular metabolism (92,93). In most cases, there is a correlation between the stimulation of amino acid uptake by a hormone in its specific target tissue

and an increase in the metabolic activities of the cell such as macromolecular synthesis.

There exists a wide variety of hormones that are capable of stimulating amino acid transport suggesting that there are several different routes by which the hormone-mediated stimulation of transport can occur (89,92). The addition of hormones may result in de novo synthesis of transport proteins. The use of the protein synthesis inhibitors puromycin or cycloheximide has shown that the action of some hormones is on the synthesis of transport proteins. The role of protein synthesis may be to produce new transport molecules or alternatively to alter the levels of factors that regulate transport activity.

The activation of inactive carriers or the recruitment of membrane transport carriers are other possible mechanisms for the hormone-mediated stimulation of amino acid transport. A second protein synthesis-independent mode of transport stimulation might occur by post-translational modification of a protein component of the transport protein (eg. protein phosphorylation).

For example, the addition of hormones insulin and glucagon can affect amino acid transport in hepatic cells in vitro and in vivo (92). Glucagon, possibly via cAMP, increased the hepatic uptake of the system A substrates AIB and MeAIB. Insulin, acting through a separate

mechanism, stimulated a transport system that had no affinity for MeAIB but increased AIB incorporation. Both of these naturally occurring hormones affected the transport of amino acids by altering the intracellular second messenger systems and both stimulated changes in membrane protein phosphorylation (92,94).

Leucine transport in human B lymphocytes is primarily via system L (56). TPA treatment of B lymphocytes from patients with chronic lymphocytic leukemia resulted in an increase in BCH-inhibitable leucine uptake (system L). The intracellular effects of TPA have been found to be mediated by the stimulation of the intracellular effector molecule, protein kinase C.

The potential toxicity of tyrosine for melanocytes make it likely that the availability of tyrosine for melanin synthesis is under stringent control. Due to the unique role of tyrosine in melanocytes and melanoma cells, it may be possible to alter its transport by altering the specific metabolic pathway of melanogenesis. Various compounds have been used to either decrease or enhance melanin synthesis <u>in vitro</u> and <u>in vivo</u>.

PTU has been used as a specific inhibitor of the enzyme tyrosinase (80). PTU inhibits tyrosinase function by entering the cells and combining directly with the enzyme resulting in the loss of tyrosinase function and a lack of pigment production in the cells. However, in

this study PTU addition did not affect tyrosine transport when compared to the untreated control (Table 3.3). Therefore, the inhibition of tyrosinase alone may not be enough to alter the tyrosine transport.

MSH has been used in a number of different melanocyte and melanoma cell lines to increase melanin production (7,11). It acts by stimulating adenylate cyclase which results in increased intracellular cAMP levels. The affects of the increased cAMP levels in pigment producing cells is not yet fully understood, but it is believed that it acts through cAMP dependent protein kinases to alter melanogenesis (7,11). Wong and Pawelek (95) were able to show that treatment of a murine melanoma cell line, Cloudman S91, with MSH resulted in an activation of pre-existing tyrosinase molecules via cAMP. When the SK-MEL 23 cells were grown in the presence of a stimulating concentration of MSH and then tested in the tyrosine transport assay, there was an increase in the tyrosine transport as compared to an untreated control cell culture (Table 3.3).

It is possible that cAMP or cAMP-dependent protein kinases are involved in the increase through the stimulation of synthesis of transporters, by increasing the number of transporters already present at the membrane, by increasing the turnover rate of these transporters already present at the membrane, or by the

recruitment of transporters not originally responsible for tyrosine transport. Any of these situations would result in an increase in the tyrosine transport in melanoma cells after stimulation with MSH.

PTU acts upon a single enzyme to attain its final effects. MSH stimulation results in the activation of the cAMP cascade which potentially could have multiple intracellular functions. The results indicate that inhibiting tyrosinase alone is not enough of a stimulus to alter cellular transport of tyrosine. It may be that more than a simple change in enzyme function is required for an alteration in transport.

4.4 POSSIBLE HORMONAL FUNCTION OF TYROSINE

Tyrosine and dopa themselves have also been suggested to act as regulators of the melanogenic apparatus in a similar fashion to that of MSH (13,18,19,96). This would mean that tyrosine and dopa may have hormone-like function in the melanocytes and melanoma cells in addition to their traditional role as an enzyme substrate. Evidence is accumulating that, in vertebrates, tyrosine and dopa can act as inducers of the melanogenic apparatus, expression and activity of MSH receptors, melanocyte proliferation, and of energy yielding metabolism (18-20). In cultured amelanotic hamster melanoma cells, the addition of tyrosine to the

growth medium induces melanogenesis and synthesis of melanogenesis proteins such as tyrosinase, structural proteins and auxiliary enzymes.

To act as a hormone, membrane receptors for these compounds would be required for the activation of an intracellular response. Tyrosine receptors have not been detected in multicellular organisms, but have been documented in unicellular organisms (97). One potential candidate as a receptor for tyrosine and dopa is the transmembrane protein tyrosinase (13). It has separate tyrosine and dopa binding sites, and melanosome specific proteins can be expressed on the cell surface. However, since tyrosinase is usually localized to the melanosome it is unlikely that there would be enough of the enzyme present on the cell surface to account for the tyrosine transport into the cell. Still tyrosinase, or other melanosomal membrane proteins, may be important for the localization of tyrosine to the site of melanin synthesis, the melanosome.

4.5 MELANOSOME AND TRANSPORT

Any transport system serving largely for amino acids of special metabolic significance in a given tissue or cell type may be expected to show some degree of functional specificity to that tissue or cell type (45). In melanocytes the special metabolic requirement is the

synthesis of melanin pigments that takes place inside the melanosome. In order for melanogenesis to occur, tyrosinase inside the melanosome must be active and the appropriate melanin precursors must be present. Consequently, it may be the melanosomal membrane that assumes ultimate control over the type of melanin synthesized by the cell. If the melanosome will not permit cysteine or sulfhydryl compounds to come into contact with the melanin intermediates, then pheomelanin cannot be synthesized. If tyrosine is incapable of entering the melanosome, then the whole process of melanogenesis may not be initiated due to a lack of substrate.

The melanosomal membrane may be responsible for the selectivity of the substrates and therefore for the type and amount of melanin produced by the cell. There are at least 15-16 polypeptides that have been identified as components of the melanosome (2). Only a few of these have been characterized as to their function, leaving many as yet undetermined with relation to their role, if any, in melanogenesis. One or more of these proteins may be associated with the regulation of melanosomal membrane transport capable of determining the nature of the melanin pigment produced by the cell and possibly involved in the control of cell growth and differentiation as a result.

System L transport has been shown to be a weakly concentrating system, unable to develop large concentration gradients across the membrane due to the wide range of compounds it transports and the exchange capabilities of this transport system (43-46). Consequently, tyrosine can gain access to the cytoplasm of melanin producing cells, but would serve as a general pool for all cellular functions. The presence of melanosomal amino acid transporters could more significantly contribute to the localization and regulation of melanin synthesis by controlling the flow of precursors into the melanosome.

The presence of these melanosomal transporters could also explain the selective accumulation of the tyrosine homolog, 4-S-CP, in the melanin synthesizing tissues. Once the compound has gained access to the inside of the cell via system L transport, melanosomal membrane proteins may allow for the transport of the compound into melanosomes. Here the metabolic machinery can metabolize the compound and therefore allow for accumulation at this location. This compartmentalization would allow for an increase in substrate concentration at the melanin synthesis site, while still allowing an adequate supply of amino acids for general cellular protein synthesis.

Future studies will need to determine the nature of the melanin precursor transport into the melanosome, and

its role, if any, in the regulation of melanin synthesis and the development of specific chemotherapeutic agents.

CHAPTER 5
CONCLUSIONS

The transport of tyrosine in the human melanoma cell line SK-MEL 23 was found to be mediated by a single, Na^+-independent transport system, system L. The K_m and V_{max} values of this transport system were 0.164 0.016 mM and 21.6 1.1 nmol/min/mg protein (mean SE) respectively. Modification of the melanin synthetic pathway, a biochemical pathway dependent on tyrosine and unique to pigment producing cells, may allow for the study of tyrosine transport regulation. Inhibition of the melanin synthesizing enzyme, tyrosinase, was not a sufficient stimulus to decrease the tyrosine transport. Treatment of the SK-MEL 23 cells with the melanogenic hormone, MSH, resulted in increased tyrosine transport. MSH has been shown to act via cAMP, which may have numerous intracellular effects that could result in increased tyrosine transport. The nature of the changes necessary for tyrosine transport regulation as well as the cause for the accumulation of tyrosine homologs in melanin producing cells are important areas for future research in the development of new anti-melanoma agents based on this unique biochemical pathway.

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